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(54) Title: METHODS AND COMPOSITIONS FOR STIMULATING BONE CELLS			
(57) Abstract			
<p>Disclosed are methods, compositions, kits and devices for use in transferring nucleic acids into bone cells <i>in situ</i> and/or for stimulating bone progenitor cells. Type II collagen and, particularly, osteotropic genes, are shown to stimulate bone progenitor cells and to promote bone growth, repair and regeneration <i>in vivo</i>. Gene transfer protocols are disclosed for use in transferring various nucleic acid materials into bone, as may be used in treating various bone-related diseases and defects including fractures, osteoporosis, osteogenesis, imperfecta and in connection with bone implants.</p>			

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- 1 -

DESCRIPTIONMethods and Compositions for Stimulating Bone Cells

5       The present application is a continuation-in-part of  
U.S. Serial Number 08/316,650, filed September 30, 1994;  
which is a continuation-in-part of U.S. Serial Number  
08/199,780, filed February 18, 1994; the entire text and  
10       figures of which disclosures are specifically  
incorporated herein by reference without disclaimer. The  
United States government has certain rights in the  
present invention pursuant to Grant HL-41926 from the  
National Institutes of Health.

15    **1. Field of the Invention**

      The present invention relates generally to the field  
of bone cells and tissues. More particularly, certain  
embodiments concern the transfer of genetic material into  
20       bone and other embodiments concern type II collagen. In  
certain examples, the invention concerns the use of type  
II collagen and nucleic acids to stimulate bone growth,  
repair and regeneration. Methods, compositions, kits and  
devices are provided for transferring an osteotropic gene  
25       into bone progenitor cells, which is shown to stimulate  
progenitor cells and to promote increased bone formation  
in vivo.

**2. Description of the Related Art**

30

      Defects in the process of bone repair and  
regeneration are linked to the development of several  
human diseases and disorders, e.g., osteoporosis and  
osteogenesis imperfecta. Failure of the bone repair  
35       mechanism is, of course, also associated with significant  
complications in clinical orthopaedic practice, for  
example, fibrous non-union following bone fracture,

- 2 -

implant interface failures and large allograft failures. The lives of many individuals would be improved by the development of new therapies designed to stimulate and strengthen the fracture repair process.

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Naturally, any new technique to stimulate bone repair would be a valuable tool in treating bone fractures. A significant portion of fractured bones are still treated by casting, allowing natural mechanisms to effect wound repair. Although there have been advances in fracture treatment in recent years, including improved devices, the development of new processes to stimulate, or complement, the wound repair mechanisms would represent significant progress in this area.

15

A very significant patient population that would benefit from new therapies designed to promote fracture repair, or even prevent or lessen fractures, are those patients suffering from osteoporosis. The term osteoporosis refers to a heterogeneous group of disorders characterized by decreased bone mass and fractures. Clinically, osteoporosis is segregated into type I and type II. Type I osteoporosis occurs predominantly in middle aged women and is associated with estrogen loss at the menopause, while osteoporosis type II is associated with advancing age.

An estimated 20-25 million people are at increased risk for fracture because of site-specific bone loss. The cost of treating osteoporosis in the United States is currently estimated to be in the order of \$10 billion per year. Demographic trends, i.e., the gradually increasing age of the US population, suggest that these costs may increase 2-3 fold by the year 2020 if a safe and



- 3 -

effective treatment is not found.

The major focus of current therapies for osteoporosis is fracture prevention, not fracture repair. This is an important consideration, as it is known that significant morbidity and mortality are associated with prolonged bed rest in the elderly, especially those who have suffered hip fracture. New methods are clearly needed for stimulating fracture repair, thus restoring mobility in these patients before the complications arise.

Osteogenesis imperfecta (OI) refers to a group of inherited connective tissue diseases characterized by bone and soft connective tissue fragility (Byers and Steiner, 1992; Prockop, 1990). Males and females are affected equally, and the overall incidence is currently estimated to be 1 in 5,000-14,000 live births. Hearing loss, dentinogenesis imperfecta, respiratory insufficiency, severe scoliosis and emphysema are just some of the conditions that are associated with one or more types of OI. While accurate estimates of the health care costs are not available, the morbidity and mortality associated with OI certainly result from the extreme propensity to fracture (OI types I-IV) and the deformation of abnormal bone following fracture repair (OI types II-IV) (Bonadio and Goldstein, 1993). The most relevant issue with OI treatment is to develop new methods by which to improve fracture repair and thus to improve the quality of life of these patients.

The techniques of bone reconstruction, such as is used to reconstruct defects occurring as a result of trauma, cancer surgery or errors in development, would also be improved by new methods to promote bone repair. Reconstructive methods currently employed, such as using autologous bone grafts, or bone grafts with attached soft

- 4 -

tissue and blood vessels, are associated with significant drawbacks of both cost and difficulty. For example, harvesting a useful amount of autologous bone is not easily achieved, and even autologous grafts often become  
5 infected or suffer from resorption.

The process of bone repair and regeneration resembles the process of wound healing in other tissues. A typical sequence of events includes; hemorrhage; clot  
10 formation; dissolution of the clot with concurrent removal of damaged tissues; ingrowth of granulation tissue; formation of cartilage; capillary ingrowth and cartilage turnover; rapid bone formation (callus tissue); and, finally, remodeling of the callus into cortical and  
15 trabecular bone. Therefore, bone repair is a complex process that involves many cell types and regulatory molecules. The diverse cell populations involved in fracture repair include stem cells, macrophages, fibroblasts, vascular cells, osteoblasts, chondroblasts,  
20 and osteoclasts.

Regulatory factors involved in bone repair are known to include systemic hormones, cytokines, growth factors, and other molecules that regulate growth and  
25 differentiation. Various osteoinductive agents have been purified and shown to be polypeptide growth-factor-like molecules. These stimulatory factors are referred to as bone morphogenetic or morphogenic proteins (BMPs), and have also been termed osteogenic bone inductive proteins  
30 or osteogenic proteins (OPs). Several BMP (or OP) genes have now been cloned, and the common designations are BMP-1 through BMP-8. New BMPs are in the process of discovery. Although the BMP terminology is widely used, it may prove to be the case that there is an OP

- 5 -

counterpart term for every individual BMP (Alper, 1994).

BMPs 2-8 are generally thought to be osteogenic, although BMP-1 is a more generalized morphogen (Shimell et al., 1991). BMP-3 is also called osteogenin (Luyten et al., 1989) and BMP-7 is also called OP-1 (Ozkaynak et al., 1990). BMPs are related to, or part of, the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily, and both TGF- $\beta$ 1 and TGF- $\beta$ 2 also regulate osteoblast function (Seitz et al., 1992). Several BMP (or OP) nucleotide sequences and polypeptides have been described in U.S. Patents, e.g., 4,795,804; 4,877,864; 4,968,590; 5,108,753; including, specifically, BMP-1 disclosed in U.S. Patent 5,108,922; BMP-2A (currently referred to as BMP-2) in U.S. Patents 5,166,058 and 5,013,649; BMP-2B (currently referred to as BMP-4) disclosed in U.S. Patent 5,013,649; BMP-3 in 5,116,738; BMP-5 in 5,106,748; BMP-6 in 5,187,076; BMP-7 in 5,108,753 and 5,141,905; and OP-1, COP-5 and COP-7 in 5,011,691.

20

Other growth factors or hormones that have been reported to have the capacity to stimulate new bone formation include acidic fibroblast growth factor (Jingushi et al., 1990); estrogen (Boden et al., 1989); macrophage colony stimulating factor (Horowitz et al., 1989); and calcium regulatory agents such as parathyroid hormone (PTH) (Raisz and Kream, 1983).

Several groups have investigated the possibility of using bone stimulating proteins and polypeptides, particularly recombinant BMPs, to influence bone repair *in vivo*. For example, recombinant BMP-2 has been employed to repair surgically created defects in the mandible of adult dogs (Toriumi et al., 1991), and high doses of this molecule have been shown to functionally repair segmental defects in rat femurs (Yasko et al., 1992). Chen and colleagues showed that a single

- 6 -

application of 25-100 mg of recombinant TGF- $\beta$ 1 adjacent to cartilage induced endochondral bone formation in the rabbit ear full thickness skin wounds (Chen et al., 1991). It has also been reported that an application of TGF- $\beta$ 1 in a 3% methylcellulose gel was able to repair surgically induced large skull defects that otherwise heal by fibrous connective tissue and never form bone (Beck et al., 1991).

However, there are many drawbacks associated with these type of treatment protocols, not least the expensive and time-consuming purification of the recombinant proteins from their host cells. Also, polypeptides, once administered to an animal are more unstable than is generally desired for a therapeutic agent, and they are susceptible to proteolytic attack. Furthermore, the administration of recombinant proteins can initiate various inhibitive or otherwise harmful immune responses. It is clear, therefore, that a new method capable of promoting bone repair and regeneration in vivo would represent a significant scientific and medical advance with immediate benefits to a large number of patients. A method readily adaptable for use with a variety of matrices and bone-stimulatory genes would be particularly advantageous.

#### SUMMARY OF THE INVENTION

The present invention overcomes one or more of these and other drawbacks inherent in the prior art by providing novel methods, compositions and devices for use in transferring nucleic acids into bone cells and tissues, and for promoting bone repair and regeneration. Certain embodiments of the invention rest, generally, with the inventors' surprising finding that nucleic acids can be effectively transferred to bone progenitor cells

- 7 -

in vivo and that, in certain embodiments, the transfer of an osteotropic gene stimulates bone repair in an animal.

The invention, in general terms, thus concerns  
5 methods, compositions and devices for transferring a nucleic acid segment into bone progenitor cells or tissues. The methods of the invention generally comprise contacting bone progenitor cells with a composition  
10 comprising a nucleic acid segment in a manner effective to transfer the nucleic acid segment into the cells. The cells may be cultured cells or recombinant cells maintained *in vitro*, when all that is required is to add the nucleic acid composition to the cells, e.g., by adding it to the culture media.

15

Alternatively, the progenitor cells may be located within a bone progenitor tissue site of an animal, when the nucleic acid composition would be applied to the site in order to effect, or promote, nucleic acid transfer  
20 into bone progenitor cells *in vivo*. In transferring nucleic acids into bone cells within an animal, a preferred method involves first adding the genetic material to a bone-compatible matrix and then using the resultant matrix to contact an appropriate tissue site  
25 within the animal. The "resultant" matrix may, in certain embodiments, be referred to as a matrix impregnated with genetic material, or it may take the form of a matrix-nucleic acid mixture, or even conjugate.

30 An extremely wide variety of genetic material can be transferred to bone progenitor cells or tissues using the compositions and methods of the invention. For example, the nucleic acid segment may be DNA (double or single-stranded) or RNA (e.g., mRNA, tRNA, rRNA); it may also be  
35 a "coding segment", i.e., one that encodes a protein or polypeptide, or it may be an antisense nucleic acid molecule, such as antisense RNA that may function to

- 8 -

disrupt gene expression. The nucleic acid segments may thus be genomic sequences, including exons or introns alone or exons and introns, or coding cDNA regions, or in fact any construct that one desires to transfer to a bone progenitor cell or tissue. Suitable nucleic acid segments may also be in virtually any form, such as naked DNA or RNA, including linear nucleic acid molecules and plasmids; functional inserts within the genomes of various recombinant viruses, including viruses with DNA genomes and retroviruses; and any form of nucleic acid segment, plasmid or virus associated with a liposome or a gold particle, the latter of which may be employed in connection with the gene gun technology.

The invention may be employed to promote expression of a desired gene in bone cells or tissues and to impart a particular desired phenotype to the cells. This expression could be increased expression of a gene that is normally expressed (i.e., "over-expression"), or it could be used to express a gene that is not normally associated with bone progenitor cells in their natural environment. Alternatively, the invention may be used to suppress the expression of a gene that is naturally expressed in such cells and tissues, and again, to change or alter the phenotype. Gene suppression may be a way of expressing a gene that encodes a protein that exerts a down-regulatory function, or it may utilize antisense technology.

#### 1. Bone Progenitor Cells and Tissues

In certain embodiments, this invention provides advantageous methods for using genes to stimulate bone progenitor cells. As used herein, the term "bone progenitor cells" refers to any or all of those cells that have the capacity to ultimately form, or contribute to the formation of, new bone tissue. This includes

- 9 -

various cells in different stages of differentiation, such as, for example, stem cells, macrophages, fibroblasts, vascular cells, osteoblasts, chondroblasts, osteoclasts, and the like. Bone progenitor cells also  
5 include cells that have been isolated and manipulated in vitro, e.g., subjected to stimulation with agents such as cytokines or growth factors or even genetically engineered cells. The particular type or types of bone  
10 progenitor cells that are stimulated using the methods and compositions of the invention are not important, so long as the cells are stimulated in such a way that they are activated and, in the context of in vivo embodiments, ultimately give rise to new bone tissue.

15       The term "bone progenitor cell" is also used to particularly refer to those cells that are located within, are in contact with, or migrate towards (i.e., "home to"), bone progenitor tissue and which cells directly or indirectly stimulate the formation of mature  
20 bone. As such, the progenitor cells may be cells that ultimately differentiate into mature bone cells themselves, i.e., cells that "directly" form new bone tissue. Cells that, upon stimulation, attract further progenitor cells or promote nearby cells to differentiate  
25 into bone-forming cells (e.g., into osteoblasts, osteocytes and/or osteoclasts) are also considered to be progenitor cells in the context of this disclosure - as their stimulation "indirectly" leads to bone repair or regeneration. Cells affecting bone formation indirectly  
30 may do so by the elaboration of various growth factors or cytokines, or by their physical interaction with other cell types. Although of scientific interest, the direct or indirect mechanisms by which progenitor cells

- 10 -

stimulate bone or wound repair is not a consideration in practicing this invention.

Bone progenitor cells and bone progenitor tissues may be cells and tissues that, in their natural environment, arrive at an area of active bone growth, repair or regeneration (also referred to as a wound repair site). In terms of bone progenitor cells, these may also be cells that are attracted or recruited to such an area. These may be cells that are present within an artificially-created osteotomy site in an animal model, such as those disclosed herein. Bone progenitor cells may also be isolated from animal or human tissues and maintained in an *in vitro* environment. Suitable areas of the body from which to obtain bone progenitor cells are areas such as the bone tissue and fluid surrounding a fracture or other skeletal defect (whether or not this is an artificially created site), or indeed, from the bone marrow. Isolated cells may be stimulated using the methods and compositions disclosed herein and, if desired, be returned to an appropriate site in an animal where bone repair is to be stimulated. In such cases, the nucleic-acid containing cells would themselves be a form of therapeutic agent. Such *ex vivo* protocols are well known to those of skill in the art.

In important embodiments of the invention, the bone progenitor cells and tissues will be those cells and tissues that arrive at the area of bone fracture or damage that one desires to treat. Accordingly, in treatment embodiments, there is no difficulty associated with the identification of suitable target progenitor cells to which the present therapeutic compositions should be applied. All that is required in such cases is to obtain an appropriate stimulatory composition, as disclosed herein, and contact the site of the bone fracture or defect with the composition. The nature of



- 11 -

this biological environment is such that the appropriate cells will become activated in the absence of any further targeting or cellular identification by the practitioner.

5        Certain methods of the invention involve, generally, contacting bone progenitor cells with a composition comprising one or more osteotropic genes (with or without additional genes, proteins or other biomolecules) so as to promote expression of said gene in said cells. As  
10        outlined above, the cells may be contacted *in vitro* or *in vivo*. This is achieved, in the most direct manner, by simply obtaining a functional osteotropic gene construct and applying the construct to the cells. The present  
15        inventors surprisingly found that there are no particular molecular biological modifications that need to be performed in order to promote effective expression of the gene in progenitor cells. Contacting the cells with DNA, e.g., a linear DNA molecule, or DNA in the form of a  
20        plasmid or other recombinant vector, that contains the gene of interest under the control of a promoter, along with the appropriate termination signals, is sufficient to result in uptake and expression of the DNA, with no further steps necessary.

25        In preferred embodiments, the process of contacting the progenitor cells with the osteotropic gene composition is conducted *in vivo*. Again, a direct consequence of this process is that the cells take up and express the gene and that they, without additional steps,  
30        function to stimulate bone tissue growth, repair or regeneration.

35        An assay of an osteoinductive gene may be conducted using the bone induction bioassay of Sampath and Reddi (1981; incorporated herein by reference). This is a rat bone formation assay that is routinely used to evaluate the osteogenic activity of bone inductive factors.

- 12 -

However, for analyzing the effects of osteotropic genes on bone growth, one is generally directed to use the novel osteotomy model disclosed herein.

## 5    2.    Osteotropic Genes

As used herein, the terms "osteotropic and osteogenic gene" are used to refer to a gene or DNA coding region that encodes a protein, polypeptide or peptide that is capable of promoting, or assisting in the promotion of, bone formation, or one that increases the rate of primary bone growth or healing (or even a gene that increases the rate of skeletal connective tissue growth or healing). The terms promoting, inducing and stimulating are used interchangeably throughout this text to refer to direct or indirect processes that ultimately result in the formation of new bone tissue or in an increased rate of bone repair. Thus, an osteotropic gene is a gene that, when expressed, causes the phenotype of a cell to change so that the cell either differentiates, stimulates other cells to differentiate, attracts bone-forming cells, or otherwise functions in a manner that ultimately gives rise to new bone tissue.

In using the new osteotomy model of the invention, an osteotropic gene is characterized as a gene that is capable of stimulating proper bone growth in the osteotomy gap to any degree higher than that observed in control studies, e.g., parallel studies employing an irrelevant marker gene such as  $\beta$ -galactosidase. This stimulation of "proper bone growth" includes both the type of tissue growth and the rate of bone formation. In using the model with a 5 mm osteotomy gap, an osteotropic gene is generally characterized as a gene that is capable of promoting or inducing new bone formation, rather than abnormal bone fracture repair, i.e., fibrous non-union. In using the 2 mm osteotomy gap, one may characterize

- 13 -

osteotropic genes as genes that increase the rate of primary bone healing as compared to controls, and more preferably, genes capable of stimulating repair of the osteotomy defect in a time period of less than nine weeks.

In general terms, an osteotropic gene may also be characterized as a gene capable of stimulating the growth or regeneration of skeletal connective tissues such as, e.g., tendon, cartilage, and ligament. Thus, in certain embodiments, the methods and compositions of the invention may be employed to stimulate the growth or repair of both bone tissue itself and also of skeletal connective tissues.

A variety of osteotropic genes are now known, all of which are suitable for use in connection with the present invention. Osteotropic genes and the proteins that they encode include, for example, systemic hormones, such as parathyroid hormone (PTH) and estrogen; many different growth factors and cytokines; chemotactic or adhesive peptides or polypeptides; molecules such as activin (U.S. Patent 5,208,219, incorporated herein by reference); specific bone morphogenetic proteins (BMPs); and even growth factor receptor genes.

Examples of suitable osteotropic growth factors include those of the transforming growth factor (TGF) gene family, including TGFs 1-3, and particularly TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3, (U.S. Patents 4,886,747 and 4,742,003, incorporated herein by reference), with TGF- $\alpha$  (U.S. Patent 5,168,051, incorporated herein by reference) also being of possible use; and also fibroblast growth factors (FGF), previously referred to as acidic and basic FGF and now referred to as FGF1-9; granulocyte/macrophage colony stimulating factor (GM-CSF); epidermal growth factor (EGF); platelet derived growth factor (PDGF); insulin-

- 14 -

like growth factors (IGF), including IGF-I and IGF-II; and leukemia inhibitory factor (LIF), also known as HILDA and DIA. Any of the above or other related genes, or DNA segments encoding the active portions of such proteins, may be used in the novel methods and compositions of the invention.

Certain preferred osteotropic genes and DNA segments are those of the TGF superfamily, such as TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3 and members of the BMP family of genes. For example, several BMP genes have been cloned that are ideal candidates for use in the nucleic acid transfer or delivery protocols of the invention. Suitable BMP genes are those designated BMP-2 through BMP-12. BMP-1 is not considered to be particularly useful at this stage.

There is considerable variation in the terminology currently employed in the literature in referring to these genes and polypeptides. It will be understood by those of skill in the art that all BMP genes that encode an active osteogenic protein are considered for use in this invention, regardless of the differing terminology that may be employed. For example, BMP-3 is also called osteogenin and BMP-7 is also called OP-1 (osteogenic protein-1). It is likely that the family of factors termed OP(s) is as large as that termed BMP(s), and that these terms, in fact, describe the same set of molecules (Alper, 1994).

The DNA sequences for several BMP (or OP) genes have been described both in scientific articles and in U.S. Patents such as 4,877,864; 4,968,590; 5,108,753. Specifically, BMP-1 sequences are disclosed in U.S. Patent 5,108,922; BMP-2A (currently referred to as BMP-2) in U.S. Patents 5,166,058 and 5,013,649; BMP-2B (currently referred to as BMP-4) disclosed in U.S. Patent 5,013,649; BMP-3 in 5,116,738; BMP-5 in 5,106,748; BMP-6

- 15 -

in 5,187,076; and BMP-7 in 5,108,753 and 5,141,905; all incorporated herein by reference). The article by Wozney et al., (1988; incorporated herein by reference) is considered to be particularly useful for describing BMP molecular clones and their activities. DNA sequences encoding the osteogenic proteins designated OP-1, COP-5 and COP-7 are also disclosed in U.S. Patent 5,011,691.

All of the above issued U.S. Patents are incorporated herein by reference and are intended to be used in order to supplement the present teachings regarding the preparation of BMP and OP genes and DNA segments that express osteotropic polypeptides. As disclosed in the above patents, and known to those of skill in the art, the original source of a recombinant gene or DNA segment to be used in a therapeutic regimen need not be of the same species as the animal to be treated. In this regard, it is contemplated that any recombinant PTH, TGF or BMP gene may be employed to promote bone repair or regeneration in a human subject or an animal, e.g., a horse. Particularly preferred genes are those from human, murine and bovine sources, in that such genes and DNA segments are readily available, with the human or murine forms of the gene being most preferred for use in human treatment regimens. Recombinant proteins and polypeptides encoded by isolated DNA segments and genes are often referred to with the prefix "r" for recombinant and "rh" for recombinant human. As such, DNA segments encoding rBMPs, such as rhBMP-2 or rhBMP-4, are contemplated to be particularly useful in connection with this invention.

The definition of a "BMP gene", as used herein, is a gene that hybridizes, under relatively stringent hybridization conditions (see, e.g., Maniatis et al., 1982), to DNA sequences presently known to include BMP gene sequences.

- 16 -

To prepare an osteotropic gene segment or cDNA one may follow the teachings disclosed herein and also the teachings of any of patents or scientific documents specifically referenced herein. Various nucleotide sequences encoding active BMPs are disclosed in U.S. Patents 5,166,058, 5,013,649, 5,116,738, 5,106,748, 5,187,076, 5,108,753 and 5,011,691, each incorporated herein by reference. By way of example only, U.S. Patent 5,166,058, teaches that hBMP-2 is encoded by a nucleotide sequence from nucleotide #356 to nucleotide #1543 of the sequence shown in Table II of the patent. One may thus obtain a hBMP-2 DNA segment using molecular biological techniques, such as polymerase chain reaction (PCR™) or screening a cDNA or genomic library, using primers or probes with sequences based on the above nucleotide sequence. The practice of such techniques is a routine matter for those of skill in the art, as taught in various scientific articles, such as Sambrook et al., (1989), incorporated herein by reference. Certain documents further particularly describe suitable mammalian expression vectors, e.g., U.S. Patent 5,168,050, incorporated herein by reference.

Osteotropic genes and DNA segments that are particularly preferred for use in certain aspects of the present compositions and methods are the TGF, PTH and BMP genes. TGF genes are described in U.S. Patents 5,168,051; 4,886,747 and 4,742,003, each incorporated herein by reference. TGF $\alpha$  may not be as widely applicable as TGF $\beta$ , but is proposed for use particularly in applications involving skeletal soft tissues. The PTH gene, or a DNA segment encoding the active fragment thereof, such as a DNA segment encoding a polypeptide that includes the amino acids 1-34 (hPTH1-34; Hendy et al., 1981; incorporated herein by reference) is another

preferred gene; as are the BMP genes termed BMP-4 and BMP-2, such as the gene or cDNA encoding the murine BMP-4 disclosed herein.

5        It is also contemplated that one may clone further genes or cDNAs that encode an osteotropic protein or polypeptide. The techniques for cloning DNA molecules, i.e., obtaining a specific coding sequence from a DNA library that is distinct from other portions of DNA, are  
10 well known in the art. This can be achieved by, for example, screening an appropriate DNA library, as disclosed in Example XV herein, which relates to the cloning of a wound healing gene. The screening procedure may be based on the hybridization of oligonucleotide  
15 probes, designed from a consideration of portions of the amino acid sequence of known DNA sequences encoding related osteogenic proteins. The operation of such screening protocols are well known to those of skill in the art and are described in detail in the scientific  
20 literature, for example, in Sambrook et al., (1989), incorporated herein by reference.

Osteotropic genes with sequences that vary from those described in the literature are also encompassed by  
25 the invention, so long as the altered or modified gene still encodes a protein that functions to stimulate bone progenitor cells in any direct or indirect manner. These sequences include those caused by point mutations, those due to the degeneracies of the genetic code or naturally  
30 occurring allelic variants, and further modifications that have been introduced by genetic engineering, i.e., by the hand of man.

Techniques for introducing changes in nucleotide  
35 sequences that are designed to alter the functional properties of the encoded proteins or polypeptides are well known in the art, e.g., U.S. Patent 4,518,584,

- 18 -

incorporated herein by reference, which techniques are also described in further detail herein. Such modifications include the deletion, insertion or substitution of bases, and thus, changes in the amino acid sequence. Changes may be made to increase the osteogenic activity of a protein, to increase its biological stability or half-life, to change its glycosylation pattern, and the like. All such modifications to the nucleotide sequences are encompassed by this invention.

It will, of course, be understood that one or more than one osteotropic gene may be used in the methods and compositions of the invention. The nucleic acid delivery methods may thus entail the administration of one, two, three, or more, osteotropic genes. The maximum number of genes that may be applied is limited only by practical considerations, such as the effort involved in simultaneously preparing a large number of gene constructs or even the possibility of eliciting a significant adverse cytotoxic effect. The particular combination of genes may be two or more distinct BMP genes; or it may be such that a growth factor gene is combined with a hormone gene, e.g., a BMP gene and a PTH gene; a hormone or growth factor gene may even be combined with a gene encoding a cell surface receptor capable of interacting with the polypeptide product of the first gene.

In using multiple genes, they may be combined on a single genetic construct under control of one or more promoters, or they may be prepared as separate constructs of the same or different types. Thus, an almost endless combination of different genes and genetic constructs may be employed. Certain gene combinations may be designed to, or their use may otherwise result in, achieving synergistic effects on cell stimulation and bone growth,



- 19 -

any and all such combinations are intended to fall within the scope of the present invention. Indeed, many synergistic effects have been described in the scientific literature, so that one of ordinary skill in the art would readily be able to identify likely synergistic gene combinations, or even gene-protein combinations.

It will also be understood that, if desired, the nucleic segment or gene could be administered in combination with further agents, such as, e.g., proteins or polypeptides or various pharmaceutically active agents. So long as genetic material forms part of the composition, there is virtually no limit to other components which may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the target cells or tissues. The nucleic acids may thus be delivered along with various other agents, for example, in certain embodiments one may wish to administer an angiogenic factor, and/or an inhibitor of bone resorption, as disclosed in U.S. Patents 5,270,300 and 5,118,667, respectively, each incorporated herein by reference.

### 3. Gene Constructs and DNA Segments

25

As used herein, the terms "gene" and "DNA segment" are both used to refer to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a gene or DNA segment encoding an osteotropic gene refers to a DNA segment that contains sequences encoding an osteotropic protein, but is isolated away from, or purified free from, total genomic DNA of the species from which the DNA is obtained. Included within the term "DNA segment", are DNA segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phage, retroviruses, adenoviruses, and the like.

- 20 -

The term "gene" is used for simplicity to refer to a functional protein or peptide encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences and cDNA sequences.

5 "Isolated substantially away from other coding sequences" means that the gene of interest, in this case, an osteotropic gene, forms the significant part of the coding region of the DNA segment, and that the DNA  
10 segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions, such as sequences encoding leader peptides or targeting  
15 sequences, later added to the segment by the hand of man.

This invention provides novel ways in which to utilize various known osteotropic DNA segments and recombinant vectors. As described above, many such  
20 vectors are readily available, one particular detailed example of a suitable vector for expression in mammalian cells is that described in U.S. Patent 5,168,050, incorporated herein by reference. However, there is no requirement that a highly purified vector be used, so  
25 long as the coding segment employed encodes a osteotropic protein and does not include any coding or regulatory sequences that would have a significant adverse effect on bone progenitor cells. Therefore, it will also be understood that useful nucleic acid sequences may include  
30 additional residues, such as additional non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, i.e., introns, which are known to occur within genes.

35 After identifying an appropriate osteotropic gene or DNA molecule, it may be inserted into any one of the many vectors currently known in the art, so that it will

- 21 -

direct the expression and production of the osteotropic protein when incorporated into a bone progenitor cell. In a recombinant expression vector, the coding portion of the DNA segment is positioned under the control of a promoter. The promoter may be in the form of the promoter which is naturally associated with an osteotropic gene, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR™ technology, in connection with the compositions disclosed herein.

In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with an osteotropic gene in its natural environment. Such promoters may include those normally associated with other osteotropic genes, and/or promoters isolated from any other bacterial, viral, eukaryotic, or mammalian cell. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in bone progenitor cells.

The use of recombinant promoters to achieve protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook et al., (1989). The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level or regulated expression of the introduced DNA segment. The currently preferred promoters are those such as CMV, RSV LTR, the SV40 promoter alone, and the SV40 promoter in combination with various enhancer elements.

Osteotropic genes and DNA segments may also be in the form of a DNA insert which is located within the genome of a recombinant virus, such as, for example a recombinant adenovirus, adeno-associated virus (AAV) or retrovirus. In such embodiments, to place the gene in contact with a bone progenitor cell, one would prepare the recombinant viral particles, the genome of which includes the osteotropic gene insert, and simply contact the progenitor cells or tissues with the virus, whereby the virus infects the cells and transfers the genetic material.

In certain preferred embodiments, one would impregnate a matrix or implant material with virus by soaking the material in recombinant virus stock solution, e.g., for 1-2 hours, and then contact the bone progenitor cells or tissues with the resultant, impregnated matrix. Cells then penetrate, or grow into, the matrix, thereby contacting the virus and allowing viral infection which leads to the cells taking up the desired gene or cDNA and expressing the encoded protein.

In other preferred embodiments, one would form a matrix-nucleic acid admixture, whether using naked DNA, a plasmid or a viral vector, and contact the bone progenitor cells or tissues with the resultant admixed matrix. The matrix may then deliver the nucleic acid into the cells following disassociation at the cell surface, or in the immediate cellular environment. Equally, the matrix admixture itself, especially a particle- or fiber-DNA admixture, may be taken up by cells to provide subsequent intracellular release of the genetic material. The matrix may then be extruded from the cell, catabolized by the cell, or even stored within the cell. The molecular mechanism by which a bone-compatible matrix achieves transfer of DNA to a cell is immaterial to the practice of the present invention.

#### 4. Bone-Compatible Matrices

In certain preferred embodiments, the methods of the invention involved preparing a composition in which the osteotropic gene, genes, DNA segments, or cells already incorporating such genes or segments, are associated with, impregnated within, or even conjugated to, a bone-compatible matrix, to form a "matrix-gene composition" and the matrix-gene composition is then placed in contact with the bone progenitor cells or tissue. The matrix may become impregnated with a gene DNA segment simply by soaking the matrix in a solution containing the DNA, such as a plasmid solution, for a brief period of time of anywhere from about 5 minutes or so, up to and including about two weeks.

Matrix-gene compositions are all those in which genetic material is adsorbed, absorbed, impregnated, conjugated to, or otherwise generally maintained in contact with the matrix. "Maintained in contact with the matrix" means that an effective amount of the nucleic acid composition should remain functionally associated with the matrix until its transfer to the bone progenitor cell or its release in the bone tissue site.

The type of matrix that may be used in the compositions, devices and methods of the invention is virtually limitless, so long as it is a "bone-compatible matrix". This means that the matrix has all the features commonly associated with being "biocompatible", in that it is in a form that does not produce a significant adverse, allergic or other untoward reaction when administered to an animal, and that it is also suitable for placing in contact with bone tissue. A "significant" adverse effect is one that exceeds the normally accepted side-effects associated with any given therapy.

"Bone-compatible", as used herein, means that the matrix (and gene) does not produce a significant adverse or untoward reaction when placed in contact with bone. In certain embodiments, when electing to use a particular bone compatible matrix, one may, optionally, take various other factors into consideration, for example, the capacity of the matrix to provide a structure for the developing bone, its capacity to be resorbed into the body after the bone has been repaired, and such like. However, these properties are not required to practice the invention and are merely exemplary of the factors that may be considered.

In other embodiments, one may also consider the likelihood that the matrix will be transported into the cell, e.g., by active or passive membrane transport. Where such transport and subsequent nucleic acid release is contemplated, other properties of the matrix and gene may be assessed in optimizing the matrix-gene formulation. For example, adenovirus vectors may provide for advantageous DNA release in such embodiments. Matrices that are readily metabolized in the cytoplasm would also likely be preferred in such embodiments. Matrices that are later released from the cell, and preferably, also removed from the surrounding tissue area, would be another preferred form of matrix for use in such embodiments.

The choice of matrix material will differ according to the particular circumstances and the site of the bone that is to be treated. Matrices such as those described in U.S. Patent 5,270,300 (incorporated herein by reference) may be employed. Physical and chemical characteristics, such as, e.g., biocompatibility, biodegradability, strength, rigidity, interface properties, and even cosmetic appearance, may be considered in choosing a matrix, as is well known to

- 25 -

those of skill in the art. Appropriate matrices will deliver the gene composition and, in certain circumstances, may be incorporated into a cell, or may provide a surface for new bone growth, i.e., they may act as an *in situ* scaffolding through which progenitor cells may migrate.

A particularly important aspect of the present invention is its use in connection with orthopaedic implants and interfaces and artificial joints, including implants themselves and functional parts of an implant, such as, e.g., surgical screws, pins, and the like. In preferred embodiments, it is contemplated that the metal surface or surfaces of an implant or a portion thereof, such as a titanium surface, will be coated with a material that has an affinity for nucleic acids, most preferably, with hydroxyl apatite, and then the coated-metal will be further coated with the gene or nucleic acid that one wishes to transfer. The available chemical groups of the absorptive material, such as hydroxyl apatite, may be readily manipulated to control its affinity for nucleic acids, as is known to those of skill in the art.

In certain embodiments, non-biodegradable matrices may be employed, such as sintered hydroxylapatite, aluminates, other bioceramic materials and metal materials, particularly titanium. A suitable ceramic delivery system is that described in U.S. Patent 4,596,574, incorporated herein by reference. Polymeric matrices may also be employed, including acrylic ester polymers, lactic acid polymers, and polylactic polyglycolic acid (PLGA) block copolymers, have been disclosed (U.S. Patent 4,526,909, U.S. Patent 4,563,489, Simons et al., 1992, and Langer and Folkman, 1976, respectively, each incorporated herein by reference).

In certain embodiments, it is contemplated that a biodegradable matrix will likely be most useful. A biodegradable matrix is generally defined as one that is capable of being resorbed into the body. Potential  
5 biodegradable matrices for use in connection with the compositions, devices and methods of this invention include, for example, biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxylapatite, PLGA block copolymers, polyanhydrides,  
10 matrices of purified proteins, and semi-purified extracellular matrix compositions.

One preferred group of matrices are collagenous matrices, including those obtained from tendon or dermal  
15 collagen, e.g., type I collagen, which is generally prepared from dermis; those obtained from cartilage, such as type II collagen; and various other types of collagen. Collagens may be obtained from a variety of commercial sources, e.g., Sigma that supplies type II collagen  
20 obtained from bovine trachea; and Collagen Corporation. Collagen matrices may also be prepared as described in U.S. Patents 4,394,370 and 4,975,527, each incorporated herein by reference.

25 The various collagenous materials may also be in the form of mineralized collagen. One preferred mineralized collagenous material is that termed UltraFiber™, obtainable from Norian Corp. (Mountain View, CA). U.S. Patent 5,231,169, incorporated herein by reference,  
30 describes the preparation of mineralized collagen through the formation of calcium phosphate mineral under mild agitation *in situ* in the presence of dispersed collagen fibrils. Such a formulation may be employed in the



- 27 -

context of delivering a nucleic acid segment to a bone tissue site.

Certain other preferred collagenous materials are those based upon type II collagen. Type II collagen preparations have been discovered to have the surprising and advantageous property of, absent any osteotropic gene, being capable of stimulating bone progenitor cells. Prior to the present invention, it was thought that type II collagen only had a structural role in the cartilage extracellular matrix and the present finding that type II collagen is actually an osteoconductive/osteoinductive material is unexpected. The present invention thus contemplates the use of a variety of type II collagen preparations as gene transfer matrices or bone cell stimulants, either with or without DNA segments, including native type II collagen, as prepared from cartilage, and recombinant type II collagen.

PLGA block copolymers may also be employed as gene transfer matrices. Such polymers have been shown to readily incorporate DNA, are commercially available, non-toxic, and hydrolyze at defined rates, (i.e. they facilitate the sustained release of pharmaceutical agents). PLGA block copolymers have two particular advantageous properties in that, first, they exhibit reversible thermal gelation, and second, may be combined with other agents that allow for radiographic visualization.

#### 5. Nucleic Acid Transfer Embodiments

Once a suitable matrix-gene composition has been prepared or obtained, all that is required to deliver the osteotropic gene to bone progenitor cells within an animal is to place the matrix-gene composition in contact

- 28 -

with the site in the body in which one wishes to promote bone growth. This may be achieved by physically positioning the matrix-gene composition in contact with the body site, or by injecting a syringeable form of the matrix-gene composition into the appropriate area.

The matrix-gene composition may be applied to a simple bone fracture site that one wishes to repair, an area of weak bone, such as in a patient with osteoporosis, or a bone cavity site that one wishes to fill with new bone tissue. Bone cavities may arise as a result of an inherited disorder, birth defect, or may result following dental or periodontal surgery or after the removal of an osteosarcoma.

The use of PLGA and like compounds as matrices allows the matrix-DNA composition to be syringeable, which is achieved by, generally, admixing the matrix-gene composition with a pluronic agent. The resultant matrix-gene-pluronic may be stored within a thermal-jacket syringe, maintained at a temperature of about 4°C, immediately prior to administration to the body. In this temperature and environment, the composition will be a liquid. Following insertion into the body, the composition will equilibrate towards body temperature, and in so-doing will form a gelatinous matrix.

The above phenomenon is termed "reversible thermal gelation", and this allows for a controlled rate of gelation to be achieved. The manner of using pluronic agents in this context will be known to those of skill in the art in light of the present disclosure. Matrix-gene-pluronic compositions may also be admixed, or generally associated with, an imaging agent so that the present gene transfer technology may be used in imaging modalities. In these cases, the attending physician or veterinarian will be able to monitor the delivery and

- 29 -

positioning of the matrix-gene composition. Many safe and effective imaging agents, such as the radiographic compound calcium phosphate, are available that may be used in conjunction with fluoroscopy, or even with tomography, to image the body or tissue site while the composition is being delivered.

Where an image of the tissue site is to be provided, one will desire to use a detectable imaging agent, such as a radiographic agent, or even a paramagnetic or radioactive agent. Many radiographic diagnostic agents are known in the art to be useful for imaging purposes, including e.g., calcium phosphate.

In the case of paramagnetic ions, examples include chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) and erbium (III), with gadolinium being generally preferred. Ions useful in other contexts, such as X-ray imaging, include but are not limited to, lanthanum (III), gold (III), lead (II), and especially bismuth (III).

Although not generally preferred, radioactive isotopes are not excluded and may be used for imaging purposes if desired. Suitable ions include iodine<sup>131</sup>, iodine<sup>123</sup>, technetium<sup>99m</sup>, indium<sup>111</sup>, rhenium<sup>188</sup>, rhenium<sup>186</sup>, gallium<sup>67</sup>, copper<sup>67</sup>, yttrium<sup>90</sup>, iodine<sup>125</sup> and astatine<sup>211</sup>.

The amount of gene construct that is applied to the matrix and the amount of matrix-gene material that is applied to the bone tissue will be determined by the attending physician or veterinarian considering various biological and medical factors. For example, one would wish to consider the particular osteotropic gene and matrix, the amount of bone weight desired to be formed,

the site of bone damage, the condition of the damaged bone, the patient's or animal's age, sex, and diet, the severity of any infection, the time of administration and any further clinical factors that may affect bone growth, such as serum levels of various factors and hormones. The suitable dosage regimen will therefore be readily determinable by one of skill in the art in light of the present disclosure, bearing in mind the individual circumstances.

10

In treating humans and animals, progress may be monitored by periodic assessment of bone growth and/or repair, e.g., using X-rays. The therapeutic methods and compositions of the invention are contemplated for use in both medical and veterinary applications, due to the lack of species specificity in bone inductive factors. In particular, it is contemplated that domestic, farm and zoological animals, as well as thoroughbred horses, would be treatable using the nucleic acid transfer protocols disclosed herein.

20

The present methods and compositions may also have prophylactic uses in closed and open fracture reduction and also in the improved fixation of artificial joints. The invention is applicable to stimulating bone repair in congenital, trauma-induced, or oncologic resection-induced craniofacial defects, and also is useful in the treatment of periodontal disease and other tooth repair processes and even in cosmetic plastic surgery. The matrix-gene compositions and devices of this invention may also be used in wound healing and related tissue repair, including, but not limited to healing of burns, incisions and ulcers.

30

The present invention also encompasses DNA-based compositions for use in cellular transfer to treat bone defects and disorders. The compositions of the invention

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generally comprise an osteotropic gene in association with a bone-compatible matrix, such as type II collagen, wherein the composition is capable of stimulating bone growth, repair or regeneration upon administration to, or  
5 implantation within, a bone progenitor tissue site of an animal. The osteotropic gene or genes may be any of those described above, with TGF- $\alpha$  (for soft skeletal tissues), TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3, PTH, BMP-2 and BMP-4 genes being generally preferred. Likewise, irrespective  
10 of the choice of gene, the bone-compatible matrix may be any of those described above, with biodegradable matrices such as collagen and, more particularly, type II collagen, being preferred.

15 In still further embodiments, the present invention concerns osteotropic devices, which devices may be generally considered as molded or designed matrix-gene compositions. The devices of the invention naturally  
20 comprise a bone-compatible matrix in which an osteotropic gene is associated with the matrix. The combination of genes and matrix components is such that the device is capable of stimulating bone growth or healing when  
25 implanted in an animal. The devices may be of virtually any size or shape, so that their dimensions are adapted to fit a bone fracture or bone cavity site in the animal that is to be treated, allowing the fracture join and/or  
bone regrowth to be more uniform. Other particularly contemplated devices are those that are designed to act  
30 as an artificial joint. Titanium devices and hydroxylapatite-coated titanium devices will be preferred in certain embodiments. Parts of devices in combination with an osteotropic nucleic acid segment, such as a DNA-coated screw for an artificial joint, and the like, also  
fall within the scope of the invention.

35

Therapeutic kits comprising, in suitable container means, a bone compatible matrix, such as type II collagen

- 32 -

or a PLGA block polymer, and an osteotropic gene form another aspect of the invention. Such kits will generally contain a pharmaceutically acceptable formulation of the matrix and a pharmaceutically acceptable formulation of an osteotropic gene, such as PTH, BMP, TGF- $\beta$ , FGF, GMCSF, EGF, PDGF, IGF or a LIF gene. Currently preferred genes include PTH, TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3, and BMP-4 genes.

10       The kits may comprise a single container means that contains both the biocompatible matrix and the osteotropic gene. The container means may, if desired, contain a pharmaceutically acceptable sterile syringeable matrix, having associated with it, the osteotropic gene composition and, optionally, a detectable label or imaging agent. The syringeable matrix-DNA formulation may be in the form of a gelatinous composition, e.g., a type II collagen-DNA composition, or may even be in a more fluid form that nonetheless forms a gel-like composition upon administration to the body. In these cases, the container means may itself be a syringe, pipette, or other such like apparatus, from which the matrix-DNA material may be applied to a bone tissue site or wound area. However, the single container means may contain a dry, or lyophilized, mixture of a matrix and osteotropic gene composition, which may or may not require pre-wetting before use.

30       Alternatively, the kits of the invention may comprise distinct container means for each component. In such cases, one container would contain the osteotropic gene, either as a sterile DNA solution or in a lyophilized form, and the other container would include the matrix, which may or may not itself be pre-wetted with a sterile solution, or be in a gelatinous, liquid or other syringeable form.

- 33 -

The kits may also comprise a second or third container means for containing a sterile, pharmaceutically acceptable buffer, diluent or solvent. Such a solution may be required to formulate either the DNA component, the matrix component, both components separately, or a pre-mixed combination of the components, into a more suitable form for application to the body, e.g., a more gelatinous form. It should be noted, however, that all components of a kit could be supplied in a dry form (lyophilized), which would allow for "wetting" upon contact with body fluids. Thus, the presence of any type of pharmaceutically acceptable buffer or solvent is not a requirement for the kits of the invention. The kits may also comprise a second or third container means for containing a pharmaceutically acceptable detectable imaging agent or composition.

The container means will generally be a container such as a vial, test tube, flask, bottle, syringe or other container means, into which the components of the kit may placed. The matrix and gene components may also be aliquoted into smaller containers, should this be desired. The kits of the present invention may also include a means for containing the individual containers in close confinement for commercial sale, such as, e.g., injection or blow-molded plastic containers into which the desired vials or syringes are retained.

Irrespective of the number of containers, the kits of the invention may also comprise, or be packaged with, an instrument for assisting with the placement of the ultimate matrix-gene composition within the body of an animal. Such an instrument may be a syringe, pipette, forceps, or any such medically approved delivery vehicle.

35

6. **Type II Collagen as an Osteoconductive/inductive Material**

The present invention also provides methods for stimulating bone progenitor cells, as may be applied, in certain circumstances, to promote new bone formation, or to stimulate wound-healing. As such, the bone progenitor cells that are the targets of the invention may also be termed "wound healing bone progenitor cells". Although the function of wound healing itself may not always be required to practice all aspects of the invention, and although a mechanistic understanding is not necessary to practice the invention, it is generally thought that the wound healing process does operate during execution of the invention.

To stimulate a bone progenitor cell in accordance with these aspects of the invention, generally one would contact a bone progenitor cell with a composition comprising a biologically effective amount of type II collagen. Although preparations of crushed bone and mineralized collagen have been shown to be osteoconductive, this property has not previously been ascribed to type II collagen. The present inventors have found that type II collagen alone is surprisingly effective at promoting new bone formation, it being able to bridge a 5 mm osteotomy gap in only eight weeks in all animals tested (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C, FIG. 6D, FIG. 7A, FIG. 7B, FIG. 8A, FIG. 8B, and FIG. 8C).

The forms of type II collagen that may be employed in this invention are virtually limitless. For example, type II collagen may be purified from hyaline cartilage of bovine trachea, or as isolated from diarthrodial joints or growth plates. Purified type II collagen is commercially available and may be purchased from, e.g., Sigma Chemical Company, St. Louis, MO. Any form of



- 35 -

recombinant type II collagen may also be employed, as may be obtained from a type II collagen-expressing recombinant host cell, including bacterial, yeast, mammalian, and insect cells. One particular example of a  
5 recombinant type II collagen expression system is a yeast cell that includes an expression vector that encodes type II collagen, as disclosed herein in Example VI.

The type II collagen used in the invention may, if  
10 desired, be supplemented with additional minerals, such as calcium, e.g., in the form of calcium phosphate. Both native and recombinant type II collagen may be supplemented by admixing, adsorbing, or otherwise associating with, additional minerals in this manner.  
15 Such type II collagen preparations are clearly distinguishable from the types of "mineralized collagen" previously described, e.g., in U.S. Patent 5,231,169 that describes the preparation of mineralized total collagen fibrils.

20  
An object of this aspect of the invention is to provide a source of osteoconductive matrix material, that may be reproducibly prepared in a straightforward and cost-effective manner, and that may be employed, with or  
25 without an osteotropic gene segment, to stimulate bone progenitor cells. Recombinant type II collagen was, surprisingly found to satisfy these criteria. Although clearly not required for effective results, the combination of native or recombinant type II collagen  
30 with mineral supplements, such as calcium, is encompassed by this invention.

A biologically effective amount of type II collagen is an amount of type II collagen that functions to  
35 stimulate a bone progenitor cell, as described herein. By way of example, one measure of a biologically effective amount is an amount effective to stimulate bone

- 36 -

progenitor cells to the extent that new bone formation is evident. In this regard, the inventors have shown that 10 mg of lyophilized collagen functions effectively to close a 5 mm osteotomy gap in three weeks. This  
5 information may be used by those of skill in the art to optimize the amount of type II collagen needed for any given situation.

Depending on the individual case, the artisan would,  
10 in light of this disclosure, readily be able to calculate an appropriate amount, or dose, of type II collagen for stimulating bone cells and promoting bone growth. In terms of small animals or human subjects, suitable effective amounts of collagen include between about 1 mg  
15 and about 500 mg, and preferably, between about 1 mg and about 100 mg, of lyophilized type II collagen per bone tissue site. Of course, it is likely that there will be variations due to, e.g., individual responses, particular tissue conditions, and the speed with which bone  
20 formation is required. While 10 mg were demonstrated to be useful in the illustrative example, the inventors contemplate that 1, 5, 10, 15, 20, 30, 40, 50, 75, 100, 125, 150, 200, 300 mg, and the like, may be usefully employed for human patients and small animals. Of  
25 course, any values within these contemplated ranges may be useful in any particular case.

Naturally, one of the main variables to be accounted for is the amount of new bone that needs to be generated  
30 in a particular area or bone cavity. This can be largely a function of the size of the animal to be treated, e.g., a cat or a horse. Therefore, there is currently no upper limit on the amount of type II collagen, or indeed on the amount of any matrix-gene composition, that can be  
35 employed in the methods of the invention, given careful supervision by the practitioner.

- 37 -

In contacting or applying type II collagen, with or without a DNA segment, to bone progenitor cells located within a bone progenitor tissue site of an animal, bone tissue growth will be stimulated. Thus, bone cavity  
5 sites and bone fractures may be filled and repaired.

The use of type II collagen in combination with a nucleic acid segment that encodes a polypeptide or protein that stimulates bone progenitor cells when  
10 expressed in said cells is preferred, as described above. Nucleic acid segments that comprise an isolated PTH gene, BMP gene, growth factor gene, growth factor receptor gene, cytokine gene or a chemotactic factor gene are preferred, with PTH, TGF- $\beta$  and BMP genes being most  
15 preferred. The genes function subsequent to their transfer into, and expression in, bone progenitor cells of the treated animal, thus promoting bone growth.

Although type II collagen alone is effective, its  
20 combined use with an osteotropic gene segment may prove to give synergistic and particularly advantageous effects. Type II collagen, whether native or recombinant, may thus also be formulated into a therapeutic kit with an osteotropic gene segment, in  
25 accordance with those kits described herein above. This includes the use of single or multiple container means, and combination with any medically approved delivery vehicle, including, but not limited to, syringes, pipettes, forceps, additional diluents, and the like.

30

#### BRIEF DESCRIPTION OF THE DRAWINGS

The drawings form part of the present specification  
35 and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings

in combination with the detailed description of specific embodiments presented herein.

FIG. 1. A model of DNA therapy for bone repair.

5

FIG. 2A. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells *in vivo*. Shown is the method of creating osteotomy and placing gene-activated matrix *in situ*.

10

FIG. 2B. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells *in vivo*. Shown is the method of fracturing repair cells where blood vessels grow into the gene-activated matrix (FIG. 2A).

15

FIG. 2C. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells *in vivo*. Shown are fractured cells taking up DNA as an episomal element, *i.e.* direct gene transfer *in vivo*.

20

FIG. 2D. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells *in vivo*. Shown are fractured repair synthesizing and secreting recombinant proteins encoded by the episomal DNA.

25

FIG. 2E. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells *in vivo*. Shown is the resulting new bone formation.

30

FIG. 3A. Achilles' tendon gene transfer is shown as a time course overview at 3 weeks post-surgery.

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- 39 -

FIG. 3B. Achilles' tendon gene transfer is shown as a time course overview at 9 weeks post-surgery.

FIG. 3C. Achilles' tendon gene transfer is shown as  
5 a time course overview at 12 weeks post-surgery.

FIG. 3D. Achilles' tendon gene transfer is shown as a time course immunohistochemistry study. Shown is the microscopy of tendon tissue that received SIS implant  
10 impregnated with expression plasmid DNA. Note the positive cytoplasmic staining of fibroblastic cells 9 weeks post-surgery.

FIG. 3E. Achilles' tendon gene transfer is shown as  
15 a time course immunohistochemistry study. Shown is the microscopy of tendon tissue that received SIS implant alone, without DNA. Note the relative absence of cytoplasmic staining.

FIG. 4. Monitoring of cruciate ligament gene  
20 transfer using a substrate utilization assay. Three weeks following the implantation of SIS soaked in a solution of the pSV40 $\beta$ -gal expression plasmid, tendon tissue was harvested, briefly fixed in 0.5%  
25 glutaraldehyde, and then incubated with X-gal according to published methods. Tissues were then embedded in paraffin and sections were cut and stained with H and E. Note the positive (arrows) staining in the cytoplasm of granulation tissues fibroblasts.

30  
FIG. 5A. Direct DNA transfer into regenerating bone:  $\beta$ -gal activity. The figure compares  $\beta$ -galactosidase activity in homogenates of osteotomy gap tissue from two Sprague-Dawley rats. In animal #1, the  
35 UltraFiber™ implant material was soaked in a solution of pSV40 $\beta$ -gal DNA, Promega) encoding bacterial  $\beta$ -galactosidase. In animal #2, the implant material was

- 40 -

soaked in a pure solution of pGL2-Promoter Vector DNA (Promega) encoding insect luciferase. Enzyme activity was determined using substrate assay kits ( $\beta$ -galactosidase and Luciferase Assay Systems, Promega).

- 5 Note that significant  $\beta$ -galactosidase activity was found only in the homogenate prepared from animal #1.

FIG. 5B. Direct DNA transfer into regenerating bone: luciferase activity. The figure compares  
10 luciferase activity in aliquots of the homogenates described in FIG. 5A. Luciferase activity was determined using the commercial reagents and protocols (Promega) described in FIG. 5A. Note that significant luciferase activity is found only in the homogenate prepared from  
15 animal #2.

FIG. 6A. Osteotomy gene transfer monitored by PTH studies. In this study an expression plasmid coding for a functional 34 amino acid peptide fragment of human  
20 parathyroid hormone (PTH1-34) was transferred and expressed in vivo using the GAM technology. The progress of new bone formation in the gap was monitored radiographically for three weeks and the animals were sacrificed. Shown is a radiograph of the osteotomy gap  
25 of the control animal that received an antisense hPTH1-34 GAM construct. There was no evidence of radiodense tissue in the gap.

FIG. 6B. Osteotomy gene transfer (FIG. 6A)  
30 monitored by PTH studies. Shown is a histological section of osteotomy repair tissue from the same control animal. The section is characterized by the presence of granulation tissue fibroblasts and capillaries.

35 FIG. 6C. Osteotomy gene transfer (FIG. 6A) monitored by PTH studies. Shown is a radiograph of the osteotomy gap that received the sense PTH1-34 GAM

- 41 -

construct. Note the presence of radiodense tissue in the gap (arrow).

**FIG. 6D. Osteotomy gene transfer (FIG. 6A)**

5 monitored by PTH studies. Shown is a histological section of osteotomy repair tissue from the same control animal. The section is characterized by the presence of trabecular bone plates that extend into the gap from the surgical margin.

10

**FIG. 7A. Osteotomy gene transfer BMP-4 studies.**

Shown is immunohistochemical evidence of BMP-4 transgene expression by granulation tissue fibroblasts near the center of an osteotomy gap three weeks after surgery.

15 Note the positive (arrows) staining of spindled cells. The BMP-4 transgene included an epitope tag (HA epitope, Pharmacia) that facilitated the identification of transgenic BMP-4 molecules. Tissue staining was performed using commercially available polyclonal anti-HA  
20 antibodies and standard procedures. Immunostaining was localized only to gap tissues. Control sections included serial sections stained with pre-immune rabbit serum and tissue sections from 13 control osteotomy gaps. In both instances all controls were negative for peroxidase  
25 staining of granulation tissue fibroblasts.

**FIG. 7B. Osteotomy gene transfer BMP-4 studies.**

Shown is the histology of newly formed bone as early as three weeks following gene transfer (FIG. 7A).

30

**FIG. 8A. Radiographic evidence of bridging new bone formation (arrows) as a consequence of BMP-4 gene transfer and expression at six weeks' post surgery. 9 and 16 weeks post-op, are presented in FIG. 8B and FIG. 8C, respectively, to demonstrate the orderly growth of new bone *in situ* over time. This animal, which has been maintained for 23 weeks, has been ambulating normally**

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- 42 -

without an external fixator for the past 7 weeks. Similar results have been obtained in a second long term animal (of two) that is now 17 weeks post-op.

5           **FIG. 8B.** Radiographic evidence of bridging new bone formation (arrows) as a consequence of BMP-4 gene transfer and expression at nine weeks' post surgery (see FIG. 8A).

10           **FIG. 8C.** Radiographic evidence of bridging new bone formation (arrows) as a consequence of BMP-4 gene transfer and expression at sixteen weeks' post surgery (see FIG. 8A).

15           **FIG. 9A.** The animal shown here is representative of the control group that received an osteotomy plus a collagen sponge without DNA of any type. The animal was maintained for 9 weeks following surgery and then sacrificed. Progress of new bone formation in the gap  
20 was monitored radiographically and histologically. Shown is a radiograph of the osteotomy gap at 9 weeks. Note the absence of radiodense tissue in the gap.

**FIG. 9B.** Shown is a histological section of  
25 osteotomy gap tissue from the control animal used in FIG 9A. The section is characterized by the presence of granulation tissue fibroblasts and capillaries.

**FIG. 10.** PLJ-HPTH1-34 expression construct. A cDNA  
30 fragment coding for a prepro-hPTH1-34 peptide was generated by PCR™ (Hendy et al., 1981) and then ligated into a *Bam*HI cloning site in the PLJ retroviral expression vector (Wilson et al., 1992). Several independent clones with the insert in the coding  
35 orientation were isolated and characterized.

**FIG. 11.** Southern analysis of retroviral



- 43 -

integration in the YZ-15 clone. 10 mg of YZ-15 genomic DNA were digested with *Kpn*I (for which there is a unique site in the vector LTR) and analyzed by Southern blotting. A cDNA fragment that coded for prepro-hPTH1-35 was used as a probe. The positive control for the Southern hybridization conditions was a *Kpn*I digest of genomic DNA from Rat-1 cells infected and selected with the recombinant, replication-defective retrovirus PLJ-hPTH1-84 (Wilson et al., 1992). *Kpn*I digests of DNA were also prepared from two negative controls: native Rat-1 cells and Rat-1 cells infected and selected with BAG ("BAG cells", (Wilson et al., 1992), a replication-defective recombinant retrovirus that encodes  $\beta$ -galactosidase, which is an irrelevant marker gene in these studies. Lane assignments were as follows: 1, PLJ-hPTH1-84 cells; 2 BAG cells; 3, YZ-15; 4, native Rat-1 cells. DNA sizes (kb) are shown at the left of the figure. As expected, a fragment of the predicted size (e.g., 4.3 kb) is seen only in lane 1 (the positive control) and in lane 3 (YZ-15 DNA).

**FIG. 12.** Northern blot analysis of a transduced Rat-1 clone. Poly-A(+)RNA was prepared from the YZ-15 clone and analyzed by Northern blotting as described (Chen et al., 1993). FIG. 12 contains two panels on a single sheet. Poly-A(+) RNA prepared from PLJ-hPTH1-84 cells, BAG cells, and native Rat-1 cells were used as positive and negative controls. Four probes were applied to a single blot following sequential stripping: hPTH1-34,  $\beta$ -gal, Neo, and  $\beta$ -actin. Lane assignments were as follows: 1, PLJ-hPTH1-84 cells; 2, BAG cells; 3, YZ-15 cells; 4, native Rat-1 cells. As expected, the hPTH1-34 transcript is seen only in lane 1 (positive control) and in lane 3-4; a Neo transcript is seen in lanes 1-3; a  $\beta$ -gal transcript is seen only in lane 2; and  $\beta$ -actin transcripts are seen in lanes 1-4.

FIG. 13. Northern analysis of poly-A(+) RNA demonstrating expression of the PTH/PTHrP receptor in osteotomy repair tissue.

5        FIG. 14. Overlapping murine cDNA clones representing the LTBP-like (LTBP-3) sequence. A partial representation of restriction sites is shown. N, NcoI; P, PvuII; R, RsaII; B, BamHI; H, HindIII. The numbering system at the bottom assumes that the "A" of the  
10 initiator Met codon is nt #1.

FIG. 15A. A schematic showing the structure of the murine fibrillin-1 gene product. Structural domains are shown below the diagram. Symbols designating various  
15 structural elements are defined in the legend to FIG. 15B.

FIG. 15B. A schematic showing the structure of the murine LTBP-like (LTBP-3) molecule. Domains #1-5 are  
20 denoted below the diagram. Symbols designate the following structural elements: EGF-CB repeats: open rectangles; TGF-bp repeats: open ovals; Fib motif: open circle; TGF-bp-like repeat: patterned oval; cysteine-rich sequences: patterned rectangles; proline/glycine-rich  
25 region: thick curved line, domain #2; proline-rich region, thick curved line, domain #3. Note that symbols designating the signal peptide have been deleted for simplicity. Additionally, the schematic assumes that EGF-like and EGF-CB repeats may extend for several amino  
30 acids beyond the C<sub>6</sub> position.

FIG. 15C. A schematic showing the structure of human LTBP-1. Domains #1-5 are denoted below the diagram. The symbols designating the structural elements  
35 are defined in the legend to FIG. 15B.

FIG. 16. Overview of expression of the new LTBP-

like (LTBP-3) gene during murine development as determined by tissue *in situ* hybridization. FIG. 16 consists of autoradiograms made by direct exposure of tissue sections to film after hybridization with radiolabeled probes. Day 8.5-9.0 sections contained embryos surrounded by intact membranes, uterine tissues, and the placental disk, cut in random planes. Day 13.5 and 16.5 sections contain isolated whole embryos sectioned in the sagittal plane near or about the mid-line. Identical conditions were maintained throughout autoradiography and photography, thereby allowing a comparison of the overall strength of hybridization in all tissue sections. The transcript is expressed in connective tissue, mesenchyme, liver, heart and CNS.

15

**FIG. 17A.** Selected microscopic views of mouse LTBP-3 gene expression in day 8.5-9.0 p.c. mouse developing tissues. All photographs in FIG. 17A- FIG. 17D were taken from the same slides used to prepare whole mount sections (after dipping slides in radiographic emulsion). Shown is the neural tube, brightfield image. 1 cm = 20 mm.

**FIG. 17B.** Selected microscopic views of mouse LTBP-3 gene expression in day 8.5-9.0 p.c. mouse developing tissues. Shown is the neural tube, darkfield image. Note expression by neuroepithelial cells and by surrounding mesenchyme. 1 cm = 20 mm.

**FIG. 17C.** Selected microscopic views of mouse LTBP-3 gene expression in day 8.5-9.0 p.c. mouse developing tissues. Shown is the heart, brightfield image. The figure demonstrates expression by myocardial and endocardial (arrowheads) cells. 1 cm = 20 mm.

35

**FIG. 17D.** Selected microscopic views of mouse LTBP-3 gene expression in day 8.5-9.0 p.c. mouse

- 46 -

developing tissues. Shown is the heart, darkfield image. The figure demonstrates expression by myocardial and endocardial (arrowheads) cells. Darkfield photomicrographs were taken after exposure of tissues to photographic emulsion for 2 weeks. In this image and the one shown in FIG. 17B, red blood cell and other plasma membranes give a faint white signal that contributes to the background of the experiment. 1 cm = 20 mm.

**FIG. 18A.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. All photographs in FIG. 18A - FIG. 18P were taken from the same slides used to prepare whole mount sections (after dipping slides in radiographic emulsion). Shown is the cartilage model of developing long bone from lower extremity, brightfield image. Expression by chondrocytes and by perichondrial cells is seen in FIG. 18B. 1 cm = 20 mm.

**FIG. 18B.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the cartilage model of developing long bone from lower extremity, darkfield image. Note expression by chondrocytes and by perichondrial cells. In all darkfield views of FIG. 18, red blood cell and other plasma membranes give a faint white signal that contributes to the background of the experiment. Note the absence of spurious hybridization signal in areas of the slide that lack cellular elements. 1 cm = 20 mm.

**FIG. 18C.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the lung, brightfield image. 1 cm = 20 mm.

**FIG. 18D.** Microscopy of mouse LTBP-3 gene

- 47 -

expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the heart, brightfield image. 1 cm = 20 mm.

5           **FIG. 18E.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the lung, darkfield image. Note expression by epithelial cells of developing airway and by the surrounding parenchymal cells. 1 cm = 20 mm.

10

**FIG. 18F.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the heart, darkfield image. Note continuing expression by myocardial cells. 1 cm = 20 mm.

15

**FIG. 18G.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the pancreas, brightfield image. 1 cm = 20 mm.

20

**FIG. 18H.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the intestine, brightfield image. 1 cm = 20 mm.

25

**FIG. 18I.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the pancreas, darkfield image. Note expression by acinar epithelial cells. 1 cm = 20 mm.

30

**FIG. 18J.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is intestine, darkfield image. Note the expression in epithelial and subepithelial cells. 1 cm = 20 mm.

35

**FIG. 18K.** Microscopy of mouse LTBP-3 gene

- 48 -

expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is kidney, brightfield image. 1 cm = 20 mm.

5        **FIG. 18L.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is skin, brightfield image. 1 cm = 20 mm.

10        **FIG. 18M.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is kidney, darkfield image. Note expression by blastemal cells beneath the kidney capsule, epithelial cells of developing nephrons and  
15        tubules, and the interstitial mesenchyme. 1 cm = 20 mm.

**FIG. 18N.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the skin, darkfield image. Note the  
20        expression by epidermal, adnexal and dermal cells of developing skin. 1 cm = 20 mm.

**FIG. 18O.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing  
25        tissues. Shown is the retina, brightfield image. 1 cm = 20 mm.

**FIG. 18P.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing  
30        tissues. Shown is the retina, darkfield image. Note expression by retinal epithelial cells and by adjacent connective tissue cells. 1 cm = 20 mm.

**FIG. 19.** Time-dependent expression of the LTBP-3  
35        gene by MC3T3-E1 cells. mRNA preparation and Northern blotting were preformed as described in Example XIV. Equal aliquots of total RNA as determined by UV

- 49 -

spectroscopy were loaded in each lane of the Northern gel. As demonstrated by UV spectroscopy were loaded in each lane of the Northern gel. As demonstrated by methylene blue staining (Sambrook et al., 1989), equal amounts of RNA were transferred to the nylon membrane. The results demonstrate a clear, strong peak in LTBP-3 gene expression by 14 days in culture. Weaker signals denoting LTBP-3 gene expression also can be observed after 5 days and 28 days in culture.

FIG. 20. Antisera #274 specifically binds LTBP-3 epitopes. Transfection of 293T cells with a full length mouse LTBP-3 expression plasmid followed by radiolabeling, preparation of medium sample, immunoprecipitation, and 4-18% gradient SDS-PAGE were performed as described in Example XIV. The figure presents a SDS-PAGE autoradiogram of medium samples following a 2 day exposure to film. Lane assignments are as follows: Lane 1, radiolabeled 293T medium (prior to transfection) immunoprecipitated with preimmune serum; Lane 2, radiolabeled 293T medium (prior to transfection) immunoprecipitated with antibody #274; Lane 3, radiolabeled 293T medium (following transfection and preincubation with 10  $\mu$ g of LTBP-3 synthetic peptide cocktail) immunoprecipitated with antibody #274; and Lane 4, radiolabeled 293T medium (following transfection) immunoprecipitated with antibody #274. As indicated by the bar, the full length LTBP-3 molecule migrated at 180-190 kDa.

FIG. 21. Co-immunoprecipitation of LTBP-3 and TGR- $\beta$ 1 produced by MC3T3-E1 cells. Aliquots ( $\sim 10^6$  incorporated CPM) of radiolabeled media produced by MC3T3-E1 cells after 7 days in culture were immunoprecipitated as described in Example XIV. Bars indicate the position of cold molecular weight standards used to estimate molecular weight (Rainbow mix,

- 50 -

Amersham). Immunoprecipitates were separated using 4%-18% gradient SDS-PAGE and reducing conditions. The figure shows a negative control lane 1 consisting of MC3T3-E1 medium immunoprecipitated with anti-LTBP-3 antibody #274. Western blotting was performed using the lower portion of the gradient gel and a commercially available antibody to TGF- $\beta$ 1 (Santa Cruz Biotechnology, Inc.). Antibody staining was detected using commercially available reagents and protocols (ECL Western Blotting Reagent, Amersham). MC3T3-E1 medium was immunoprecipitated with anti-LTBP-2 antibody #274.

FIG. 22A. Radiographic analysis of the type II collagen osteotomy gap three weeks after surgery.

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FIG. 22B. Radiographic analysis of the type I collagen osteotomy gap three weeks after surgery.

FIG. 22C. Histologic analysis of the type II collagen osteotomy shown in FIG. 22A.

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FIG. 23A. Adenovirus-mediated gene transfer into bone repair/regeneration cells *in vivo*. Positive (arrows)  $\beta$ -gal cytoplasmic staining is observed in the fracture repair cells.

25

FIG. 23B. Adenovirus-mediated gene transfer into bone repair/regeneration cells *in vivo*. Serial section negative control stained with the vehicle of the  $\beta$ -gal antibody plus a cocktail of non-specific rabbit IgG antibodies.

30

FIG. 23C. Adenovirus-mediated gene transfer into bone repair/regeneration cells *in vivo*. Osteotomy site was filled with a fibrous collagen implant material soaked in a solution of the replication-defective recombinant adenovirus AdRSV $\beta$ -gal ( $\sim 10^{11}$  plaque forming

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- 51 -

units/ml). Note the positive (arrow)  $\beta$ -gal nuclear staining of chondrocytes within the osteotomy site, as demonstrated by immunohistochemistry using a specific anti- $\beta$ -gal antibody.

5

**FIG. 24.** The murine BMP-4 amino acid sequence, SEQ ID NO:1. The HA epitope is shown in bold at the extreme carboxy terminus of the sequence.

10 **FIG. 25.** DNA sequence of the murine LTBP-3 gene (SEQ ID NO:2).

**FIG. 26.** Amino acid sequence of the murine LTBP-3 gene product (SEQ ID NO:3).

15

**FIG. 27.** DNA sequence of the murine LTBP-2 gene (SEQ ID NO:17).

20 **FIG. 28.** Amino acid sequence of the murine LTBP-2 gene product (SEQ ID NO:18).

#### DESCRIPTION OF THE PREFERRED EMBODIMENT

25 **1. Applications of Bone Repair Technology to Human Treatment**

The following is a brief discussion of four human conditions to exemplify the variety of diseases and disorders that would benefit from the development of new technology to improve bone repair and healing processes. In addition to the following, several other conditions, such as, for example, vitamin D deficiency; wound healing in general; soft skeletal tissue repair; and cartilage and tendon repair and regeneration, may also benefit from technology concerning the stimulation of bone progenitor cells.

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The first example is the otherwise healthy individual who suffers a fracture. Often, clinical bone fracture is treated by casting to alleviate pain and allow natural repair mechanisms to repair the wound.

5 While there has been progress in the treatment of fracture in recent times, even without considering the various complications that may arise in treating fractured bones, any new procedures to increase bone healing in normal circumstances would represent a great

10 advance.

A second example which may benefit from new treatment methods is osteogenesis imperfecta (OI). OI encompasses various inherited connective tissue diseases

15 that involve bone and soft connective tissue fragility in humans (Byers and Steiner, 1992; Prockop, 1990). About one child per 5,000-20,000 born is affected with OI and the disease is associated with significant morbidity throughout life. A certain number of deaths also occur,

20 resulting in part from the high propensity for bone fracture and the deformation of abnormal bone after fracture repair (OI types II-IV; Bonadio and Goldstein, 1993). The relevant issue here is quality of life; clearly, the lives of affected individuals would be

25 improved by the development of new therapies designed to stimulate and strengthen the fracture repair process.

OI type I is a mild disorder characterized by bone fracture without deformity, blue sclerae, normal or near

30 normal stature, and autosomal dominant inheritance (Bonadio and Goldstein, 1993). Osteopenia is associated with an increased rate of lone bone fracture upon ambulation (the fracture frequency decreases dramatically at puberty and during young adult life, but increases

35 once again in late middle age). Hearing loss, which often begins in the second or third decade, is a feature of this disease in about half the families and can

- 53 -

progress despite the general decline in fracture frequency. Dentinogenesis imperfecta is observed in a subset of individuals.

5           In contrast, OI types II-VI represent a spectrum of more severe disorders associated with a shortened life-span. OI type II, the perinatal lethal form, is characterized by short stature, a soft calvarium, blue sclerae, fragile skin, a small chest, floppy appearing  
10 lower extremities (due to external rotation and abduction of the femurs), fragile tendons and ligaments, bone fracture with severe deformity, and death in the perinatal period due to respiratory insufficiency. Radiographic signs of bone weakness include compression  
15 of the femurs, bowing of the tibiae, broad and beaded ribs, and calvarial thinning.

          OI type III is characterized by short stature, a triangular facies, severe scoliosis, and bone fracture  
20 with moderate deformity. Scoliosis can lead to emphysema and a shortened life-span due to respiratory insufficiency. OI type IV is characterized by normal sclerae, bone fracture with mild to moderate deformity, tooth defects, and a natural history that essentially is  
25 intermediate between OI type II and OI type I.

          More than 200 OI mutations have been characterized since 1989 (reviewed in Byers and Steinert, 1992; Prockop, 1990). The vast majority occur in the COL1A1 and COL1A2  
30 genes of type I collagen. Most cases of OI type I appear to result from heterozygous mutations in the COL1A1 gene that decrease collagen production but do not alter primary structure, i.e., heterozygous null mutations affecting COL1A1 expression. Most cases of OI types II-  
35 IV result from heterozygous mutations in the COL1A1 and COL1A2 genes that alter the structure of collagen.

- 54 -

A third important example is osteoporosis. The term osteoporosis refers to a heterogeneous group of disorders characterized by decreased bone mass and fractures. An estimated 20-25 million people are at increased risk for fracture because of site-specific bone loss. Risk factors for osteoporosis include increasing age, gender (more females), low bone mass, early menopause, race (Caucasians), low calcium intake, reduced physical activity, genetic factors, environmental factors (including cigarette smoking and abuse of alcohol or caffeine), and deficiencies in neuromuscular control that create a propensity to fall.

More than a million fractures in the USA each year can be attributed to osteoporosis, and in 1986 alone the treatment of osteoporosis cost an estimated 7-10 billion health care dollars. Demographic trends (i.e., the gradually increasing age of the US population) suggest that these costs may increase 2-3 fold by the year 2020 if a safe and effective treatment is not found. Clearly, osteoporosis is a significant health care problem.

Clinically, osteoporosis is segregated into type I and type II. Type I osteoporosis occurs predominantly in middle aged women and is associated with estrogen loss at the menopause, while osteoporosis type II is associated with advancing age. Much of the morbidity and mortality associated with osteoporosis results from immobilization of elderly patients following fracture.

Current therapies for osteoporosis patients focus on fracture prevention, not fracture repair. This remains an important consideration because of the literature, which clearly states that significant morbidity and mortality are associated with prolonged bed rest in the elderly, particularly those who have suffered hip fractures. Complications of bed rest include blood clots

- 55 -

and pneumonia. These complications are recognized and measures are usually taken to avoid them, but these measures hardly represent the best approach to therapy. Thus, the osteoporotic patient population would benefit from new therapies designed to strengthen bone and speed up the fracture repair process, thereby getting these people on their feet before the complications arise.

A fourth example is related to bone reconstruction and, specifically, the ability to reconstruct defects in bone tissue that result from traumatic injury; cancer or cancer surgery; birth defect; a developmental error or heritable disorder; or aging. There is a significant orthopaedic need for more stable total joint implants, and cranial and facial bone are particular targets for this type of reconstructive need. The availability of new implant materials, e.g., titanium, has permitted the repair of relatively large defects. Titanium implants provide excellent temporary stability across bony defects. However, experience has shown that a lack of viable bone bridging the defect can result in exposure of the appliance, infection, structural instability and, ultimately, failure to repair the defect.

Autologous bone grafts are another possible reconstructive modality, but they have several demonstrated disadvantages in that they must be harvested from a donor site such as iliac crest or rib, they usually provide insufficient bone to completely fill the defect, and the bone that does form is sometimes prone to infection and resorption. Partially purified xenogeneic preparations are not practical for clinical use because microgram quantities are purified from kilograms of bovine bone, making large scale commercial production both costly and impractical. Allografts and demineralized bone preparations are therefore often employed.

- 56 -

Microsurgical transfers of free bone grafts with attached soft tissue and blood vessels can close bony defects with an immediate source of blood supply to the graft. However, these techniques are time consuming, have been shown to produce a great deal of morbidity, and can only be used by specially trained individuals. Furthermore, the bone implant is often limited in quantity and is not readily contoured. In the mandible, for example, the majority of patients cannot wear dental appliances using presently accepted techniques (even after continuity is established), and thus gain little improvement in the ability to masticate. Toriumi et al., have written that, "reconstructive surgeons should have at their disposal a bone substitute that would be reliable, biocompatible, easy to use, and long lasting and that would restore mandibular continuity with little associated morbidity."

In connection with bone reconstruction, specific problem areas for improvement are those concerned with treating large defects, such as created by trauma, birth defects, or particularly, following tumor resection. The success of orthopaedic implants, interfaces and artificial joints could conceivably be improved if the surface of the implant, or a functional part of an implant, were to be coated with a bone stimulatory agent. The surface of implants could be coated with one or more appropriate materials in order to promote a more effective interaction with the biological site surrounding the implant and, ideally, to promote tissue repair.

## 2. Bone Repair

Bone tissue is known to have the capacity for repair and regeneration and there is a certain understanding of the cellular and molecular basis of these processes. The

- 57 -

initiation of new bone formation involves the commitment, clonal expansion, and differentiation of progenitor cells. Once initiated, bone formation is promoted by a variety of polypeptide growth factors. Newly formed bone  
5 is then maintained by a series of local and systemic growth and differentiation factors.

The concept of specific bone growth-promoting agents is derived from the work of Huggins and Urist. Huggins  
10 et al., 1936, demonstrated that autologous transplantation of canine incisor tooth to skeletal muscle resulted in local new bone formation (Huggins et al., 1936). Urist and colleagues reported that  
15 demineralized lyophilized bone segments induced bone formation (Urist, 1965; Urist et al., 1983), a process that involved macrophage chemotaxis; the recruitment of progenitor cells; the formation of granulation tissue, cartilage, and bone; bone remodeling; and marrow  
20 differentiation. The initiation of cartilage and bone formation in an extraskeletal site, a process referred to as osteoinduction, has permitted the unequivocal identification of initiators of bone morphogenesis (Urist, 1965; Urist et al., 1983; Sampath et al., 1984; Wang et al., 1990; Cunningham et al., 1992).

25 Significant progress has now been made in characterizing the biological agents elaborated by active bone tissue during growth and natural bone healing. Demineralized bone matrix is highly insoluble; Sampath  
30 and Reddi (1981) showed that only 3% of the proteins can be extracted using strong combinations of denaturants and detergents. They also showed that the unfractionated demineralized bone extract will initiate bone  
morphogenesis, a critical observation that led to the  
35 purification of "osteoinductive" molecules. Families of proteinaceous osteoinductive factors have now been purified and characterized. They have been variously

- 58 -

referred to in the literature as bone morphogenetic or morphogenic proteins (BMPs), osteogenic bone inductive proteins or osteogenic proteins (OPs).

5     **3. Bone Repair and Bone Morphogenetic Proteins (BMPs)**

Following their initial purification, several bone morphogenetic protein genes have now been cloned using molecular techniques (Wozney et al., 1988; Rosen et al.,  
10     1989; summarized in Alper, 1994). This work has established BMPs as members of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily based on DNA sequence homologies. Other TGF molecules have also been shown to participate in new bone formation, and TGF- $\beta$  is regarded  
15     as a complex multifunctional regulator of osteoblast function (Centrella et al., 1988; Carrington et al., 1988; Seitz et al., 1992). Indeed, the family of transforming growth factors (TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3) has been proposed as potentially useful in the treatment  
20     of bone disease (U.S. Patent 5,125,978, incorporated herein by reference).

The cloning of distinct BMP genes has led to the designation of individual BMP genes and proteins as BMP-1  
25     through BMP-8. BMPs 2-8 are generally thought to be osteogenic (BMP-1 may be a more generalized morphogen; Shimell et al., 1991). BMP-3 is also called osteogenin (Luyten et al., 1989) and BMP-7 is also called OP-1 (Ozkaynak et al., 1990). TGFs and BMPs each act on cells  
30     via complex, tissue-specific interactions with families of cell surface receptors (Roberts and Sporn, 1989; Paralkar et al., 1991).

Several BMP (or OP) nucleotide sequences and  
35     vectors, cultured host cells and polypeptides have been described in the patent literature. For example, U.S. Patents, 4,877,864, 4,968,590 and 5,108,753 all concern



- 59 -

osteogenic factors. More specifically, BMP-1 is disclosed in U.S. Patent 5,108,922; BMP-2 species, including MBP-2A and BMP-2B, are disclosed in U.S. Patents 5,166,058, 5,013,649, and 5,013,649; BMP-3 in 5,116,738; BMP-5 in 5,106,748; BMP-6 in 5,187,076; and BMP-7 in 5,108,753 and 5,141,905; all incorporated herein by reference. Various BMP clones and their activities are particularly described by Wozney et al., (1988; incorporated herein by reference). DNA sequences encoding the osteogenic proteins designated OP-1, COP-5 and COP-7 are also disclosed in U.S. Patent 5,011,691. Although the BMP terminology is widely used, it may prove to be the case that there is an OP counterpart term for every individual BMP (Alper, 1994).

15

#### 4. Bone Repair and Growth Factors and Cytokines

Transforming growth factors (TGFs) have a central role in regulating tissue healing by affecting cell proliferation, gene expression, and matrix protein synthesis (Roberts and Sporn, 1989). While not necessarily a direct effect, Bolander and colleagues have provided evidence that TGF- $\beta$ 1 and TGF- $\beta$ 2 can initiate both chondrogenesis and osteogenesis (Joyce et al., 1990; Izumi et al., 1992; Jingushi et al., 1992). In these studies new cartilage and bone formation appeared to be dose dependent (i.e., dependent on the local growth factor concentration). The data also suggested that TGF- $\beta$ 1 and TGF- $\beta$ 2 stimulated cell differentiation by a similar mechanism, even though they differed in terms of the ultimate amount of new cartilage and bone that was formed.

Other growth factors/hormones besides TGF and BMP may influence new bone formation following fracture. Bolander and colleagues injected recombinant acidic fibroblast growth factor into a rat fracture site

- 60 -

(Jingushi et al., 1990). The major effect of multiple high doses (1.0 mg/50 ml) was a significant increase in cartilage tissue in the fracture gap, while lower doses had no effect. These investigators also used the reverse transcriptase-polymerase chain reaction (PCR™) technique to demonstrate expression of estrogen receptor transcripts in callus tissue (Boden et al., 1989). These results suggested a role for estrogen in normal fracture repair.

10

Horowitz and colleagues have shown that activated osteoblasts will synthesize the cytokine, macrophage colony stimulating factor (Horowitz et al., 1989). The osteotropic agents used in this study included lipopolysaccharide, PTH1-84, PTH1-34, vitamin D and all-trans retinoic acid. This observation has led to the suggestion that osteoblast activation following fracture may lead to the production of cytokines that regulate both hematopoiesis and new bone formation. Various other proteins and polypeptides that have been found to be expressed at high levels in osteogenic cells, such as, e.g., the polypeptide designated Vgr-1 (Lyons et al., 1989), also have potential for use in connection with the present invention.

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## 25 5. Bone Repair and Calcium Regulating Hormones

Calcium regulating hormones such as parathyroid hormone (PTH) participate in new bone formation and bone remodeling (Raisz and Kream, 1983). PTH is an 84 amino acid calcium-regulating hormone whose principle function is to raise the  $\text{Ca}^{+2}$  concentration in plasma and extracellular fluid. Studies with the native hormone and with synthetic peptides have demonstrated that the amino-terminus of the molecule (aa 1-34) contains the structural requirements for biological activity (Tregear et al., 1973; Hermann-Erlee et al., 1976; Riond, 1993).

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- 61 -

PTH functions by binding to a specific cell surface receptor that belongs to the G protein-coupled receptor superfamily (Silve et al., 1982; Rizzoli et al., 1983; Juppner et al., 1991).

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Using a retroviral approach, a human full-length PTH gene construct has been introduced into cultured rat fibroblasts to create recombinant PTH-secreting cells. These cells were then transplanted into syngeneic rat recipients that were observed to develop hypercalcemia mediated by the increased serum concentrations of PTH (Wilson et al., 1992). The object of these studies was to create an animal model of primary hyperparathyroidism.

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PTH has a dual effect on new bone formation, a somewhat confusing aspect of hormone function despite intensive investigation. PTH has been shown to be a potent direct inhibitor of type I collagen production by osteoblasts (Kream et al., 1993). Intact PTH was also shown to stimulate bone resorption in organ culture over 30 years ago, and the hormone is known to increase the number and activity of osteoclasts. Recent studies by Gay and colleagues have demonstrated binding of [<sup>125</sup>I]PTH(1-84) to osteoclasts in tissue sections and that osteoclasts bind intact PTH in a manner that is both saturable and time- and temperature dependent (Agarwala and Gay, 1992). While these properties are consistent with the presence of PTH/PTHrP receptors on the osteoclast cell surface, this hypothesis is still considered controversial. A more accepted view, perhaps, is that osteoclast activation occurs via an osteoblast signaling mechanism.

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On the other hand, osteosclerosis may occur in human patients with primary hyperparathyroidism (Seyle, 1932). It is well known that individuals with hyperparathyroidism do not inexorably lose bone mass, but

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- 62 -

eventually achieve a new bone remodeling steady state after an initial period of net bone loss. Chronic, low dose administration of the amino-terminal fragment of PTH (aa 1-34) also can induce new bone formation according to  
5 a time- and dose-dependent schedule (Seyle, 1932; Parsons and Reit, 1974).

Human PTH1-34 has recently been shown to: stimulate DNA synthesis in chick osteoblasts and chondrocytes in  
10 culture (van der Plas, 1985; Schluter et al., 1989; Somjen et al., 1990); increase bone cell number *in vivo* (Malluche et al., 1986); enhance the *in vitro* growth of chick embryonic cartilage and bone (Kawashima, 1980; Burch and Lebovitz, 1983; Lewinson and Silbermann, 1986;  
15 Endo et al., 1980; Klein-Nulend et al., 1990); enhance surface bone formation (both cortical and trabecular bone) in normal and osteogenic animals and in humans with osteoporosis (Reeve et al., 1976; Reeve et al., 1980; Tam et al., 1982; Hefti et al., 1982; Podbesek et al., 1983;  
20 Stevenson and Parsons, 1983; Slovik et al., 1986; Gunness-Hey and Hock, 1984; Tada et al., 1988; Spencer et al., 1989; Hock and Fonseca, 1990; Liu and Kalu, 1990; Hock and Gera, 1992; Mitlak et al., 1992; Ejersted et al., 1993); and delay and reverse the catabolic effects  
25 of estrogen deprivation on bone mass (Hock et al., 1988; Hori et al., 1988; Gunness-Hey and Hock, 1989; Liu et al., 1991). Evidence of synergistic interactions between hPTH-1-34 and other anabolic molecules has been presented, including insulin-like growth factor, BMP-2,  
30 growth hormone, vitamin D, and TGF- $\beta$  (Slovik et al., 1986; Spencer et al., 1989; Mitlak et al., 1992; Canalis et al., 1989; Linkhart and Mohan, 1989; Seitz et al., 1992; Vukicevic et al., 1989).

35 Anecdotal observation has shown that serum PTH levels may be elevated following bone fracture (Meller et al., 1984; Johnston et al., 1985; Compston et al., 1989;

- 63 -

Hardy et al., 1993), but the significance of this observation is not understood. There are apparently no reports in the literature concerning attempts to localize either PTH or the PTH/PTHrP receptor *in situ* in human fracture sites or in experimental models. Furthermore, no attempt has been made to augment bone repair by the exogenous addition of PTH peptides. Although hPTH1-34 is known to function as an anabolic agent for bone, prior to the present invention, much remained to be learned about the role (if any) of PTH during bone regeneration and repair.

#### 6. Protein Administration and Bone Repair

Several studies have been conducted in which preparations of protein growth factors, including BMPs, have been administered to animals in an effort to stimulate bone growth. The results of four such exemplary studies are described blow.

Toriumi et al., studied the effect of recombinant BMP-2 on the repair of surgically created defects in the mandible of adult dogs (Toriumi et al., 1991). Twenty-six adult hounds were segregated into three groups following the creation of a 3 cm full thickness mandibular defect: 12 animals received test implants composed of inactive dog bone matrix carrier and human BMP-2, 10 animals received control implants composed of carrier without BMP-2, and BMP-4 animals received no implant. The dogs were euthanized at 2.5-6 months, and the reconstructed segments were analyzed by radiography, histology, histomorphometry, and biomechanical testing. Animals that received test implants were euthanized after 2.5 months because of the presence of well mineralized bone bridging the defect. The new bone allowed these animals to chew a solid diet, and the average bending strength of reconstructed mandibles was 27% of normal

- 64 -

('normal' in this case represents the unoperated, contralateral hemimandible). In contrast, the implants in the other two groups were non-functional even after 6 months and showed minimal bone formation.

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Yasko et al., published a related study in which the effect of BMP-2 on the repair of segmental defects in the rat femur was examined (Yasko et al., 1992). The study design included a group that received a dose of 1.4 mg of BMP-2, another group that received 11.0 mg of BMP-2, and a control group that received carrier matrix alone. Endochondral bone formation was observed in both groups of animals that received BMP-2. As demonstrated by radiography, histology, and whole bone (torsion) tests of mechanical integrity, the larger dose resulted in functional repair of the 5-mm defect beginning 4.5 weeks after surgery. The lower dose resulted in radiographic and histological evidence of new bone formation, but functional union was not observed even after 9 weeks post surgery. There was also no evidence of bone formation in control animals at this time.

Chen et al., showed that a single application of 25-100 mg of recombinant TGF- $\beta$ 1 adjacent to cartilage induced endochondral bone formation in the rabbit ear full thickness skin wounds (Chen et al., 1991). Bone formation began 21 days following the creation of the wound and reached a peak at day 42, as demonstrated by morphological methods. Active bone remodeling was observed beyond this point.

In a related study, Beck et al., demonstrated that a single application of TGF- $\beta$ 1 in a 3% methylcellulose gel was able to repair surgically induced large skull defects that otherwise heal by fibrous connective tissue and never form bone (Beck et al., 1991). Bony closure was achieved within 28 days of the application of 200 mg of

- 65 -

TGF- $\beta$ 1 and the rate of healing was shown to be dose dependent.

Studies such as those described above have thus  
5 established that exogenous growth factors can be used to  
stimulate new bone formation/repair/regeneration in vivo.  
Certain U.S. Patents also concern methods for treating  
bone defects or inducing bone formation. For example,  
U.S. Patent 4,877,864 relates to the administration of a  
10 therapeutic composition of bone inductive protein to  
treat cartilage and/or bone defects; U.S. Patent  
5,108,753 concerns the use of a device containing a pure  
osteogenic protein to induce endochondral bone formation  
and for use in periodontal, dental or craniofacial  
15 reconstructive procedures.

However, nowhere in this extensive literature does  
there appear to be any suggestion that osteogenic genes  
themselves may be applied to an animal in order to  
20 promote bone repair or regeneration. Indeed, even  
throughout the patent literature that concerns genes  
encoding various bone stimulatory factors and their in  
vitro expression in host cells to produce recombinant  
proteins, there seems to be no mention of the possibility  
25 of using nucleic acid transfer in an effort to express an  
osteogenic gene in bone progenitor cells in vivo or to  
promote new bone formation in an animal or human subject.

#### 7. Biocompatible Matrices for use in Bone Repair

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There is a considerable amount of work that has been  
directed to the development of biocompatible matrices for  
use in medical implants, including those specifically for  
bone implantation work. In context of the present  
35 invention, a matrix may be employed in association with  
the gene or DNA coding region encoding the osteotropic  
polypeptide in order to easily deliver the gene to the

- 66 -

site of bone damage. Such matrices may be formed from a variety of materials presently in use for implanted medical applications.

5           In certain cases, the matrix may also act as a "biofiller" to provide a structure for the developing bone and cartilage. However, the formation of such a scaffolding structure is not a primary requirement, rather, the main requirements of the matrix are to be  
10       biocompatible and to be capable of delivering a nucleic acid segment to a bone cell or bone tissue site.

          Matrices that may be used in certain embodiments include non-biodegradable and chemically defined  
15       matrices, such as sintered hydroxyapatite, bioglass, aluminates, and other ceramics. The bioceramics may be altered in composition, such as in calcium-aluminate-phosphate; and they may be processed to modify particular physical and chemical characteristics, such as pore size,  
20       particle size, particle shape, and biodegradability. Certain polymeric matrices may also be employed if desired, these include acrylic ester polymers and lactic acid polymers, as disclosed in U.S. Patents 4,526,909, and 4,563,489, respectively, each incorporated herein by  
25       reference. Particular examples of useful polymers are those of orthoesters, anhydrides, propylene-cofumarates, or a polymer of one or more  $\alpha$ -hydroxy carboxylic acid monomers, e.g.,  $\alpha$ -hydroxy acetic acid (glycolic acid) and/or  $\alpha$ -hydroxy propionic acid (lactic acid).

30           Some of the preferred matrices for use in present purposes are those that are capable of being resorbed into the body. Potential biodegradable matrices for use in bone gene transfer include, for example, PLGA block  
35       copolymers, biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxyapatite, and polyanhydrides. Furthermore, biomatrices comprised of



- 67 -

pure proteins and/or extracellular matrix components may be employed.

5 The inventors have shown the use of bone or dermal collagenous materials as matrices, as may be prepared from various commercially-available lyophilized collagen preparations, such as those from bovine or rat skin, as well as PLGA block copolymers. Collagen matrices may also be formulated as described in U.S. Patent 4,394,370,  
10 incorporated herein by reference, which concerns the use of collagenous matrices as delivery vehicles for osteogenic protein. UltraFiber™, as may be obtained from Norian Corp. (Mountain View, CA), is a preferred matrix. Preferred matrices are those formulated with type II  
15 collagen, and most preferably, recombinant type II collagen and mineralized type II collagen.

Further suitable matrices may also be prepared from combinations of materials, such as PLGA block copolymers,  
20 which allow for sustained release; hydroxyapatite; or collagen and tricalciumphosphate. Although sufficient sequestration and subsequent delivery of an osteotropic gene is in no way a limitation of the present invention, should it be desired, a porous matrix and gene  
25 combination may also be administered to the bone tissue site in combination with an autologous blood clot. The basis for this is that blood clots have previously been employed to increase sequestration of osteogenic proteins for use in bone treatment (U.S. Patent 5,171,579,  
30 incorporated herein by reference) and their use in connection with the present invention is by no means excluded (they may even attract growth factors for cytokines).

## 8. Collagen

Although not previously proposed for use with a nucleic acid molecule, the use of collagen as a pharmaceutical delivery vehicle has been described. The biocompatibility of collagen matrices is well known in the art. U.S. Patents 5,206,028, 5,128,136, 5,081,106, 4,585,797, 4,390,519, and 5,197,977 (all incorporated herein by reference) describe the biocompatibility of collagen-containing matrices in the treatment of skin lesions, use as a wound dressing, and as a means of controlling bleeding. In light of these documents, therefore, there is no question concerning the suitability of applying a collagen preparation to a tissue site of an animal.

U.S. Patent 5,197,977 describes the preparation of a collagen-impregnated vascular graft including drug materials complexed with the collagen to be released slowly from the graft following implant. U.S. Patent 4,538,603 is directed to an occlusive dressing useful for treating skin lesions and a granular material capable of interacting with wound exudate. U.S. Patent 5,162,430 describes a pharmaceutically acceptable, non-immunogenic composition comprising a telopeptide collagen chemically conjugated to a synthetic hydrophilic polymer.

Further documents that one of skill in the art may find useful include U. S. Patents 4,837,285, 4,703,108, 4,409,332, and 4,347,234, each incorporated herein by reference. These references describe the uses of collagen as a non-immunogenic, biodegradable, and bioresorbable binding agent.

The inventors contemplate that collagen from many sources will be useful in the present invention. Particularly useful are the amino acid sequences of type

- 69 -

II collagen. Examples of type II collagen are well known in the art. For example, the amino acid sequences of human (Lee et al., 1989), rat (Michaelson et al., 1994), and murine (Ortman et al., 1994) have been determined  
5 (SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:14, respectively).

Although not previously known to be capable of stimulating bone progenitor cells itself, type II  
10 collagen is herein surprisingly shown to possess this property, which thus gives rise to new possibilities for clinical uses.

## 9. Nucleic Acid Delivery

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The transfer of nucleic acids to mammalian cells has been proposed a method for treating certain diseases or disorders. Nucleic acid transfer or delivery is often referred to as "gene therapy". Initial efforts toward  
20 postnatal (somatic) gene therapy relied on indirect means of introducing genes into tissues, e.g., target cells were removed from the body, infected with viral vectors carrying recombinant genes, and implanted into the body. These type of techniques are generally referred to as ex  
25 vivo treatment protocols. Direct in vivo gene transfer has recently been achieved with formulations of DNA trapped in liposomes (Ledley et al., 1987); or in proteoliposomes that contain viral envelope receptor proteins (Nicolau et al., 1983); calcium phosphate-  
30 coprecipitated DNA (Benvenisty and Reshef, 1986); and DNA coupled to a polylysine-glycoprotein carrier complex (Wu and Wu, 1988). The use of recombinant replication-defective viral vectors to infect target cells in vivo has also been described (e.g., Seeger et al., 1984).

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In recent years, Wolff et al., demonstrated that direct injection of purified preparations of DNA and RNA

- 70 -

into murine skeletal muscle resulted in significant reporter gene expression (Wolff et al., 1990). This was an unexpected finding, and the mechanism of gene transfer could not be defined. The authors speculated that muscle  
5 cells may be particularly suited to take up and express polynucleotides in vivo or that damage associated with DNA injection may allow transfection to occur.

Wolff et al., suggested several potential  
10 applications of the direct injection method, including (a) the treatment of heritable disorders of muscle, (b) the modification of non-muscle disorders through muscle tissue expression of therapeutic transgenes, (c) vaccine development, and (d) a reversible type of gene transfer,  
15 in which DNA is administered much like a conventional pharmaceutical treatment. In an elegant study Liu and coworkers recently showed that the direct injection method can be successfully applied to the problem of influenza vaccine development (Ulmer et al., 1993).

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The use of gene transfer to synoviocytes as a means of treating arthritis has also been discussed (Bandara et al., 1992; Roessler et al., 1993). The protocols considered have included both the ex vivo treatment of  
25 isolated synoviocytes and their re-introduction into the animal and also direct gene transfer in which suitable vectors are injected into the joint. The transfer of marker genes into synoviocytes has already been demonstrated using both retroviral and adenoviral  
30 technology (Bandara et al., 1992; Roessler et al., 1993).

Despite the exclusive emphasis on protein treatment by those working in the field of new bone growth, the present inventors saw that there was great potential for  
35 using nucleic acids themselves to promote bone regeneration/repair in vivo. This provides for a more sophisticated type of pharmaceutical delivery. In

- 71 -

addition to the ease and cost of preparing DNA, it was also reasoned that using DNA transfer rather than peptide transfer would provide many further advantages. For example, DNA transfer allows for the expression or over-expression of integral membrane receptors on the surface of bone regeneration/repair cells, whereas this cannot be done using peptide transfer because the latter (*a priori*) is an extracellular manipulation. Importantly, DNA transfer also allows for the expression of polypeptides modified in a site-directed fashion with the very minimum of additional work (*i.e.*, straightforward molecular biological manipulation without protein purification) as well as sustained release of therapies delivered by an injectable route.

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The advantages of using DNA are also manifold regarding the development of pharmaceutical products and effective means of delivery. Here, important advantages include the ability to prepare injectable formulations, especially those compositions that exhibit reversible thermal gelation, and the opportunity to combine such injectables with imaging technology during delivery. "Sustained release" is also an important advantage of using DNA, in that the exogenously added DNA continues to direct the production of a protein product following incorporation into a cell. The use of certain matrix-DNA compositions also allows for a more typical "sustained release" phenomenon in that the operative release of DNA from the matrix admixture can also be manipulated.

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The inventors contemplated that both naked DNA and viral-mediate DNA could be employed in an effort to transfer genes to bone progenitor cells. In beginning to study this, the most appropriate animal model had to be employed, that is, one in which the possibilities of using nucleic acids to promote bone repair could be adequately tested in controlled studies.

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## 10. Osteotomy Model

Prior to the present invention, three model systems were available for study in this area, including Mov13 mice, an animal model of OI. Unfortunately, each of the models suffers from significant drawbacks. With the Mov13 mice, first, these mice typically die in young adulthood because of retrovirus-induced leukemia (Schnieke et al., 1983); second, gene transfer studies in Mov13 mice conducted between postnatal weeks 8-16 (*i.e.*, prior to the development of leukemia) may be complicated by a natural adaptation in which a significant amount of new bone is deposited on the periosteal surface (Bonadio et al., 1993); and third, an osteotropic gene transferred into an osteotomy site may synergize with the active retrovirus and make it even more virulent.

Another system is the *in vivo* bone fracture model created by Einhorn and colleagues (Bonnarens and Einhorn, 1984). However, this model is a closed system that would not easily permit initial studies of gene transfer *in vivo*. The organ culture model developed by Bolander and colleagues (Joyce et al., 1990) was also available, but again, this model is not suitable for studying gene transfer *in vivo*. Due to the unsuitability of the above models for studying the effects of gene transfer on bone repair and regeneration, the inventors employed a rat osteotomy system, as described below.

The important features of the rat osteotomy model are as follows: under general anesthesia, four 1.2 mm diameter pins are screwed into the femoral diaphysis of normal adult Sprague-Dawley rats. A surgical template ensures parallel placement of the pins. An external fixator is then secured on the pins, and a 2 mm, or 5 mm, segmental defect is created in the central diaphysis with a Hall micro 100 oscillating saw. A biodegradable

- 73 -

implant material, soaked in a solution of plasmid DNA, other genetic construct or recombinant virus preparation, is then placed in the intramedullary canal and the defect is closed (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C, FIG. 6D, FIG. 7A, FIG. 7B, FIG. 8A, FIG. 8B, FIG. 8C).

New bone formation can be detected as early as three weeks later in the 2 mm gap, although up to 9 weeks is generally allowed for new bone formation to occur. The fixator provided the necessary stability, and there were no limitations on animal ambulation. The surgical protocol has been successfully performed on 21/21 animals to date. None of these animals have died. Assays of new bone formation are performed after sacrifice, except plain film radiography, which is performed weekly from the time of surgery to sacrifice.

Previous studies in Sprague-Dawley rats have shown that the 5 mm osteotomy gap will heal as a fibrous non-union, whereas a gap of less than 3 mm, (such as the 2 mm gap routinely employed in the studies described herein) will heal by primary bone formation. Studies using the 5 mm gap thus allow a determination of whether transgene expression can stimulate new bone formation when fibrous tissue healing normally is expected. On the other hand, studies with the 2 mm gap allow a determination of whether transgene expression can speed up natural primary bone healing. Controls were also performed in which animals received no DNA (FIG. 9A and FIG. 9B).

#### 11. Gene Transfer Promotes Bone Repair In Vivo

The present inventors surprisingly found that gene transfer into bone progenitor cells *in vivo* (i.e., cells in the regenerating tissue in the osteotomy gap) could be readily achieved. Currently, the preferred methods for achieving gene transfer generally involve using a fibrous

- 74 -

collagen implant material soaked in a solution of DNA shortly before being placed in the site in which one desires to promote bone growth. As the studies presented herein show, the implant material facilitates the uptake of exogenous plasmid constructs by cells (in the osteotomy gap) which clearly participate in bone regeneration/repair. The transgenes, following cellular uptake, direct the expression of recombinant polypeptides, as evidenced by the *in vivo* expression of functional marker gene products.

Further studies are presented herein demonstrating that the transfer of an osteotropic gene results in cellular expression of a recombinant osteotropic molecule, which expression is directly associated with stimulation of new bone formation. After considering a relatively large number of candidate genes, a gene transfer vector coding for a fragment of human parathyroid hormone (hPTH1-34) was chosen for the inventors' initial studies. Several factors were considered in making this choice: (a), recombinant hPTH1-34 peptides can be discriminated from any endogenous rat hormone present in osteotomy tissues; (b), hPTH1-34 peptides will stimulate new bone formation in Sprague-Dawley rats, indicating that the human peptide can efficiently bind the PTH/PTHrP receptor on the rat osteoblast cell surface; and (c), there is only one PTH/PTHrP receptor, the gene for this receptor has been cloned, and cDNA probes to the receptor are available.

30

Thus, in terms of understanding the mechanism of action of the transgene on new bone formation *in vivo*, the inventors reasoned it most straightforward to correlate the expression of recombinant hPTH1-34 peptide and its receptor with new bone formation in the rat osteotomy model. Of course, following these initial studies, it is contemplated that any one of a wide

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- 75 -

variety of genes may be employed in connection with the bone gene transfer embodiments of the present invention.

Previous studies have indicated that hPTH1-34 is a  
5 more powerful anabolic agent when given intermittently as  
opposed to continuously. Despite the fact that an  
anabolic effect would still be expected with continuous  
dosing, as documented by the studies of Parsons and co-  
workers (Tam et al., 1982; Spencer et al., 1989), there  
10 was a concern that the PLJ-hPTH1-34 transgene may not  
function very effectively as transfected cells would be  
expected to express recombinant hPTH1-34 molecules in a  
constitutive manner. The finding that transfection and  
expression of the LPH-hPTH1-34 transgene did effectively  
15 stimulate bone formation in the rat osteotomy model was  
therefore an important result.

As the osteotomy site in this model is highly  
vascularized, one possible complication of the studies  
20 with the PLJ-hPTH1-34 transgene is the secretion of  
recombinant human PTH from the osteotomy site with  
consequent hypercalcemia and (potentially) animal death.  
Weekly serum calcium levels should therefore be  
determined when using this transgene. The fact that no  
25 evidence of disturbed serum calcium levels has been found  
in this work is therefore a further encouraging finding.

These studies complement others by the inventors in  
which direct gene transfer was employed to introduce  
30 genes into Achilles' tendon and cruciate ligament, as  
described in Example XI.

Various immediate applications for using nucleic  
acid delivery in connection with bone disorders became  
35 apparent to the inventors following these surprising  
findings. The direct transfer of an osteotropic gene to  
promote fracture repair in clinical orthopaedic practice

- 76 -

is just one use. Other important aspects of this technology include the use of gene transfer to treat patients with "weak bones", such as in diseases like osteoporosis; to improve poor healing which may arise for unknown reasons, e.g., fibrous non-union; to promote implant integration and the function of artificial joints; to stimulate healing of other skeletal tissues such as Achilles' tendon; and as an adjuvant to repair large defects. In all such embodiments, DNA is being used as a direct pharmaceutical agent.

## 12. Biological Functional Equivalents

As mentioned above, modification and changes may be made in the structure of an osteotropic gene and still obtain a functional molecule that encodes a protein or polypeptide with desirable characteristics. The following is a discussion based upon changing the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. The amino acid changes may be achieved by changing the codons of the DNA sequence, according to the following codon table:

- 77 -

Table 1

Amino Acids			Codons							
	Alanine	Ala	A	GCA	GCC	GCG	GCU			
	Cysteine	Cys	C	UGC	UGU					
5	Aspartic acid	Asp	D	GAC	GAU					
	Glutamic acid	Glu	E	GAA	GAG					
	Phenylalanine	Phe	F	UUC	UUU					
	Glycine	Gly	G	GGA	GGC	GGG	GGU			
	Histidine	His	H	CAC	CAU					
10	Isoleucine	Ile	I	AUA	AUC	AUU				
	Lysine	Lys	K	AAA	AAG					
	Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU	
	Methionine	Met	M	AUG						
	Asparagine	Asn	N	AAC	AAU					
15	Proline	Pro	P	CCA	CCC	CCG	CCU			
	Glutamine	Gln	Q	CAA	CAG					
	Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU	
	Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU	
	Threonine	Thr	T	ACA	ACC	ACG	ACU			
20	Valine	Val	V	GUA	GUC	GUG	GUU			
	Tryptophan	Trp	W	UGG						
	Tyrosine	Tyr	Y	UAC	UAU					

25           For example, certain amino acids may be substituted  
for other amino acids in a protein structure without  
appreciable loss of interactive binding capacity with  
structures such as, for example, antigen-binding regions  
of antibodies or binding sites on substrate molecules.

30       Since it is the interactive capacity and nature of a  
protein that defines that protein's biological functional  
activity, certain amino acid sequence substitutions can  
be made in a protein sequence, and, of course, its  
underlying DNA coding sequence, and nevertheless obtain a

35       protein with like properties. It is thus contemplated by  
the inventors that various changes may be made in the DNA  
sequences of osteotropic genes without appreciable loss  
of their biological utility or activity.

- 78 -

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporate herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: Isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, i.e., still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within  $\pm 2$  is preferred, those which are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the

- 79 -

greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

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As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0  $\pm$  1); glutamate (+3.0  $\pm$  1); serine (+0.3);  
10 asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5  $\pm$  1); alanine (-0.5); histidine \*-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

15

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In  
20 such changes, the substitution of amino acids whose hydrophilicity values are within  $\pm 2$  is preferred, those which are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred.

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As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of  
30 the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

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### 13. Site-Specific Mutagenesis

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by various publications. As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a

- 81 -

double stranded vector which includes within its sequence a DNA sequence which encodes the desired osteotropic protein. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically.

5 This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes

10 the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

15 The preparation of sequence variants of the selected osteotropic gene using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are

20 other ways in which sequence variants of osteotropic genes may be obtained. For example, recombinant vectors encoding the desired osteotropic gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

25

#### 14. Monoclonal Antibody Generation

Means for preparing and characterizing antibodies are well known in the art (See, e.g., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogenic composition in accordance with the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically the animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster, a guinea pig or a goat. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As is also well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred



- 83 -

adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*), incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

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The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to  
10 administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster injection, may  
15 also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate MAbs.

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MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable  
25 animal with a selected immunogen composition, e.g., a purified or partially purified LTBP-3 protein, polypeptide or peptide. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are  
30 preferred animals, however, the use of rabbit, sheep frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986, pp. 60-61), but mice are preferred, with the BALB/c mouse being most preferred

- 84 -

as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately  $5 \times 10^7$  to  $2 \times 10^8$  lymphocytes.

20

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render them incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

30

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, pp. 65-66, 1986; Campbell, pp. 75-83, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and

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- 85 -

4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the NS-1 myeloma cell line (also termed P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by requesting cell line repository number GM3573. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described by Kohler and Milstein (1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter et al. (1977). The use of electrically induced fusion methods is also appropriate (Goding pp. 71-74, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, about  $1 \times 10^{-6}$  to  $1 \times 10^{-8}$ . However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine

- 86 -

synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

5

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, e.g., hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

10  
15

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

20  
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The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide MAbs. The cell lines may be exploited for MAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal

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- 87 -

antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MABs in high concentration. The individual cell lines could also be cultured in vitro, where the MABs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. MABs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

### 15. LTBP-3

Other aspects of the present invention concern isolated DNA segments and recombinant vectors encoding LTBP-3, and the creation and use of recombinant host cells through the application of DNA technology, that express LTBP-3 gene products. As such, the invention concerns DNA segment comprising an isolated gene that encodes a protein or peptide that includes an amino acid sequence essentially as set forth by a contiguous sequence from SEQ ID NO:3. These DNA segments are represented by those that include a nucleic acid sequence essentially as set forth by a contiguous sequence from SEQ ID NO:2 (FIG. 25). Compositions that include a purified protein that has an amino acid sequence essentially as set forth by the amino acid sequence of SEQ ID NO:3 (FIG. 26) are also encompassed by the invention.

30

The TGF- $\beta$ s represent a family of structurally related molecules with diverse effects on mammalian cell shape, growth, and differentiation (Roberts and Sporn, 1990). Initially synthesized as a precursor consisting of an amino-terminal propeptide followed by mature TGF- $\beta$ , two chains of nascent pro-TGF- $\beta$  associate in most tissues to form a Mr ~106,000 inactive disulfide-bonded dimer.

Homodimers are most common, but heterodimers have also been described (Cheifetz et al., 1987; Ogawa et al., 1992). During biosynthesis the mature TGF- $\beta$  dimer is cleaved from the propeptide dimer. TGF- $\beta$  latency results in part from the non-covalent association of propeptide and mature TGF- $\beta$  dimers (Pircher et al., 1984, 1986; Wakefield et al., 1987; Millan et al., 1992; Miyazono and Heldin, 1989). Consequently, the propeptide dimer is often referred to as the latency associated protein (LAP), and LAP plus the disulfide-bonded TGF- $\beta$  dimer are also known as the small latent complex. In the extracellular space small latent complexes must be dissociated to activate mature TGF- $\beta$ . The mechanism of activation of the latent complex is thought to be one of the most important steps governing TGF- $\beta$  effects (Lyons et al., 1988; Antonelli-Orlidge et al., 1989; Twardzik et al., 1990; Sato et al., 1993).

In certain lines of cultured cells small latent growth factor complexes may contain additional high molecular weight proteins. The best characterized of these high molecular weight proteins is the latent TGF- $\beta$  binding protein, or LTBP (Miyazono et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990; Olofsson et al., 1992; Taketazu et al., 1994). LTBP produced by different cell types is heterogeneous in size, perhaps because of alternative splicing or because of tissue-specific proteolytic processing (Miyazono et al., 1988; Wakefield et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990). Latent TGF- $\beta$  complexes that contain LTBP are known as large latent complexes. LTBP has no known covalent linkage to mature TGF- $\beta$ , but rather it is linked by a disulfide bond to LAP.

Regarding the novel protein LTBP-3, the present invention concerns DNA segments, that can be isolated from virtually any mammalian source, that are free from  
5 total genomic DNA and that encode proteins having LTBP-3-like activity. DNA segments encoding LTBP-3-like species may prove to encode proteins, polypeptides, subunits, functional domains, and the like.

10 As used herein, the term "DNA segment" refers to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a DNA segment encoding LTBP-3 refers to a DNA segment that contains  
15 LTBP-3 coding sequences yet is isolated away from, or purified free from, total genomic DNA of the species from which the DNA segment is obtained. Included within the term "DNA segment", are DNA segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phagemids,  
20 phage, viruses, and the like.

Similarly, a DNA segment comprising an isolated or purified LTBP-3 gene refers to a DNA segment including  
25 LTBP-3 coding sequences and, in certain aspects, regulatory sequences, isolated substantially away from other naturally occurring genes or protein encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein, polypeptide or peptide encoding unit. As will be understood by those  
30 in the art, this functional term includes both genomic sequences, cDNA sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides or peptides.

35 "Isolated substantially away from other coding sequences" means that the gene of interest, in this case, a gene encoding LTBP-3, forms the significant part of the

- 90 -

coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

In particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences that encode an LTBP-3 species that includes within its amino acid sequence an amino acid sequence essentially as set forth in SEQ ID NO:3. In other particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences that include within their sequence a nucleotide sequence essentially as set forth in SEQ ID NO:2.

The term "a sequence essentially as set forth in SEQ ID NO:3" means that the sequence substantially corresponds to a portion of SEQ ID NO:3 and has relatively few amino acids that are not identical to, or a biologically functional equivalent of, the amino acids of SEQ ID NO:3. The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein (for example, see section 7, preferred embodiments). Accordingly, sequences that have between about 70% and about 80%; or more preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids that are identical or functionally equivalent to the amino acids of SEQ ID NO:3 will be sequences that are "essentially as set forth in SEQ ID NO:3".

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In certain other embodiments, the invention concerns isolated DNA segments and recombinant vectors that



- 91 -

include within their sequence a nucleic acid sequence essentially as set forth in SEQ ID NO:2. The term "essentially as set forth in SEQ ID NO:2" is used in the same sense as described above and means that the nucleic acid sequence substantially corresponds to a portion of SEQ ID NO:2 and has relatively few codons that are not identical, or functionally equivalent, to the codons of SEQ ID NO:2. Again, DNA segments that encode proteins exhibiting LTBP-3-like activity will be most preferred.

10

It will also be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5' or 3' sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, i.e., introns, which are known to occur within genes.

25

Naturally, the present invention also encompasses DNA segments that are complementary, or essentially complementary, to the sequence set forth in SEQ ID NO:2. Nucleic acid sequences that are "complementary" are those that are capable of base-pairing according to the standard Watson-Crick complementarity rules. As used herein, the term "complementary sequences" means nucleic acid sequences that are substantially complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic acid segment of SEQ ID NO:2,

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under relatively stringent conditions such as those described herein.

5       The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall  
10       length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, nucleic  
15       acid fragments may be prepared that include a short contiguous stretch identical to or complementary to SEQ ID NO:2, such as about 14 nucleotides, and that are up to about 10,000 or about 5,000 base pairs in length, with segments of about 3,000 being preferred in certain cases.  
20       DNA segments with total lengths of about 1,000, about 500, about 200, about 100 and about 50 base pairs in length (including all intermediate lengths) are also contemplated to be useful.

25       It will be readily understood that "intermediate lengths", in these contexts, means any length between the quoted ranges, such as 14, 15, 16, 17, 18, 19, 20, etc.; 21, 22, 23, etc.; 30, 31, 32, etc.; 50, 51, 52, 53, etc.; 100, 101, 102, 103, etc.; 150, 151, 152, 153, etc.;  
30       including all integers through the 200-500; 500-1,000; 1,000-2,000; 2,000-3,000; 3,000-5,000; 5,000-10,000 ranges, up to and including sequences of about 12,001, 12,002, 13,001, 13,002 and the like.

35       It will also be understood that this invention is not limited to the particular nucleic acid and amino acid sequences of SEQ ID NO:2 and SEQ ID NO:3. Recombinant

- 93 -

vectors and isolated DNA segments may therefore variously include the LTBP-3 coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region, or they may encode larger polypeptides that nevertheless include LTBP-3-coding regions or may encode biologically functional equivalent proteins or peptides that have variant amino acid sequences.

10       The DNA segments of the present invention encompass biologically functional equivalent LTBP-3 proteins and peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, 15       functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by 20       man may be introduced through the application of site-directed mutagenesis techniques, e.g., to introduce improvements to the antigenicity of the protein or to test mutants in order to examine activity at the 25       molecular level.

      If desired, one may also prepare fusion proteins and peptides, e.g., where the LTBP-3 coding regions are aligned within the same expression unit with other 30       proteins or peptides having desired functions, such as for purification or immunodetection purposes (e.g., proteins that may be purified by affinity chromatography and enzyme label coding regions, respectively).

35       Recombinant vectors form further aspects of the present invention. Particularly useful vectors are contemplated to be those vectors in which the coding

portion of the DNA segment, whether encoding a full length protein or smaller peptide, is positioned under the control of a promoter. The promoter may be in the form of the promoter that is naturally associated with a LTBP-3 gene, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR™ technology, in connection with the compositions disclosed herein.

10

In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with an LTBP-3 gene in its natural environment. Such promoters may include LTBP-3 promoters normally associated with other genes, and/or promoters isolated from any bacterial, viral, eukaryotic, or mammalian cell. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook et al., 1989. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins or peptides. Appropriate promoter systems contemplated for use in high-level expression include, but are not limited

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- 95 -

to, the *Pichia* expression vector system (Pharmacia LKB Biotechnology) (see Example XVI herein).

5 In connection with expression embodiments to prepare recombinant LTBP-3 proteins and peptides, it is contemplated that longer DNA segments will most often be used, with DNA segments encoding the entire LTBP-3 protein or functional domains, subunits, etc. being most preferred. However, it will be appreciated that the use  
10 of shorter DNA segments to direct the expression of LTBP-3 peptides or epitopic core regions, such as may be used to generate anti-LTBP-3 antibodies, also falls within the scope of the invention. DNA segments that encode peptide antigens from about 15 to about 50 amino acids in length,  
15 or more preferably, from about 15 to about 30 amino acids in length are contemplated to be particularly useful.

The LTBP-3 gene and DNA segments may also be used in connection with somatic expression in an animal or in the  
20 creation of a transgenic animal. Again, in such embodiments, the use of a recombinant vector that directs the expression of the full length or active LTBP-3 protein is particularly contemplated.

25 In addition to their use in directing the expression of the LTBP-3 protein, the nucleic acid sequences disclosed herein also have a variety of other uses. For example, they also have utility as probes or primers in nucleic acid hybridization embodiments. As such, it is  
30 contemplated that nucleic acid segments that comprise a sequence region that consists of at least a 14 nucleotide long contiguous sequence that has the same sequence as, or is complementary to, a 14 nucleotide long contiguous sequence of SEQ ID NO:2 will find particular utility.  
35 Longer contiguous identical or complementary sequences, e.g., those of about 20, 30, 40, 50, 100, 200, 500, 1000

- 96 -

(including all intermediate lengths) and even up to full length sequences will also be of use in certain embodiments.

5           The ability of such nucleic acid probes to specifically hybridize to LTBP-3-encoding sequences will enable them to be of use in detecting the presence of complementary sequences in a given sample. However, other uses are envisioned, including the use of the  
10           sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

          Nucleic acid molecules having sequence regions  
15           consisting of contiguous nucleotide stretches of 10-14, 15-20, 30, 50, or even of 100-200 nucleotides or so, identical or complementary to SEQ ID NO:2, are particularly contemplated as hybridization probes for use in, e.g., Southern and Northern blotting. This would  
20           allow LTBP-3 structural or regulatory genes to be analyzed, both in diverse cell types and also in various mammalian cells. The total size of fragment, as well as the size of the complementary stretch(es), will ultimately depend on the intended use or application of  
25           the particular nucleic acid segment. Smaller fragments will generally find use in hybridization embodiments, wherein the length of the contiguous complementary region may be varied, such as between about 10-14 and about 100 nucleotides, but larger contiguous complementarity  
30           stretches may be used, according to the length complementary sequences one wishes to detect.

          The use of a hybridization probe of about 10-14 nucleotides in length allows the formation of a duplex  
35           molecule that is both stable and selective. Molecules having contiguous complementary sequences over stretches greater than 10 bases in length are generally preferred,

though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-  
5 complementary stretches of 15 to 20 contiguous nucleotides, or even longer where desired.

Hybridization probes may be selected from any portion of any of the sequences disclosed herein. All  
10 that is required is to review the sequence set forth in SEQ ID NO:2 and to select any continuous portion of the sequence, from about 10-14 nucleotides in length up to and including the full length sequence, that one wishes to utilize as a probe or primer. The choice of probe and  
15 primer sequences may be governed by various factors, such as, by way of example only, one may wish to employ primers from towards the termini of the total sequence.

The process of selecting and preparing a nucleic acid segment that includes a contiguous sequence from within SEQ ID NO:2 may alternatively be described as preparing a nucleic acid fragment. Of course, fragments may also be obtained by other techniques such as, e.g., by mechanical shearing or by restriction enzyme  
20 digestion. Small nucleic acid segments or fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, as is commonly practiced using an automated oligonucleotide synthesizer. Also, fragments may be obtained by  
30 application of nucleic acid reproduction technology, such as the PCR™ technology of U.S. Patent 4,603,102 (incorporated herein by reference), by introducing selected sequences into recombinant vectors for recombinant production, and by other recombinant DNA  
35 techniques generally known to those of skill in the art of molecular biology.

Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of LTBP-3 gene or cDNA fragments. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, e.g., one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of 50°C to 70°C. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating LTBP-3 genes.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate LTBP-3-encoding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions will typically be needed in order to allow formation of the heteroduplex. In these circumstances, one may desire to employ conditions such as about 0.15 M to about 0.9 M salt, at temperatures ranging from 20°C to 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.



- 99 -

In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one will likely desire to employ a fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, etc.). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantitated, by means of the label.

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The following examples are included to demonstrate preferred embodiments of the invention. It should be

- 100 -

appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus  
5 can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result  
10 without departing from the spirit and scope of the invention.

#### EXAMPLE I

##### ANIMAL MODEL FOR ASSESSING NEW BONE FORMATION

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As various animal models were not suitable for studying the effects of nucleic acid transfer on bone formation, the inventors employed the following model system. The important features of the rat osteotomy  
20 model are as described in the following protocol (which is generally completed in 25-35 minutes).

The osteotomy was performed on one femur per animal. Right to left differences have not been apparent, but  
25 such differences are monitored in these studies, since the limb receiving the osteotomy is randomized.

After pre-operative preparation (i.e., shaving and Betadine® scrub), adult male Sprague Dawley rats (~500  
30 gm, retired male breeders) were anesthetized using a 3% halothane 97% oxygen mixture (700 ml/min. flow rate). A lateral approach to the femur was made on one limb. Utilizing specially designed surgical guides, four 1.2-mm diameter pins were screwed into the diaphysis after pre-  
35 drilling with a high speed precision bit. A surgical template ensured precise and parallel placement of the pins. The order of pin placement was always the same:

- 101 -

outer proximal first and then outer distal, inner proximal and inner distal (with "outer" and "inner" referring to the distance from the hip joint). Pin placement in the center of the femur was ensured by  
5 fluoroscopic imaging during pin placement. The external fixator was secured on the pins and a 1 mm or 2 mm segmental defect was created in the central diaphysis through an incision using a Hall Micro 100 Oscillating saw (#5053-60 Hall surgical blades) under constant  
10 irrigation. Other than the size of the segmental defect, there is no difference between the 5 mm and 2 mm osteotomy protocols (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C, FIG. 6D, FIG. 7A, FIG. 7B, FIG. 8A, FIG. 8B, FIG. 8C).

15

The contents of the osteotomy site were irrigated with sterile saline and the fibrous collagen implant material, previously soaked in a solution of plasmid DNA or other DNA construct, if appropriate, was placed in  
20 *situ*. The wound was then closed in layers. Since the fixator provided the necessary stability no limitations on animal ambulation existed, and other supports were not required. The surgical protocol has been successfully performed on 53 animals to date, including 35 controls  
25 (Table 2 and FIG. 24). None of these animals have died and no significant adverse effects have been observed, other than complications that might be associated with surgical fracture repair. Minor complications that were experienced include 1 animal that developed a post-  
30 operative osteomyelitis and 1 animal in which 2/4 pins loosened as a consequence of post-operative bone fracture.

- 102 -

## EXAMPLE II

IMPLANT MATERIAL FOR USE IN BONE GENE TRANSFER

Various implant materials may be used for  
5 transferring genes into the site of bone repair and/or  
regeneration in vivo. These materials are soaked in a  
solution containing the DNA or gene that is to be  
transferred to the bone regrowth site. Alternatively,  
DNA may be incorporated into the matrix as a preferred  
10 method of making.

One particular example of a suitable material is  
fibrous collagen, which may be lyophilized following  
extraction and partial purification from tissue and then  
15 sterilized. A particularly preferred collagen is the  
fibrous collagen implant material termed UltraFiber™, as  
may be obtained from Norian Corp., (Mountain View, CA).  
Detailed descriptions of the composition and use of  
UltraFiber™ are provided in Gunasekaran et al., (1993a,  
20 b; each incorporated herein by reference).

A more particularly preferred collagen is type II  
collagen, with most particularly preferred collagen being  
either recombinant type II collagen, or mineralized type  
25 II collagen. Prior to placement in osteotomy sites,  
implant materials are soaked in solutions of DNA (or  
virus) under sterile conditions. The soaking may be for  
any appropriate and convenient period, e.g., from 6  
minutes to over-night. The DNA (e.g., plasmid) solution  
30 will be a sterile aqueous solution, such as sterile water  
or an acceptable buffer, with the concentration generally  
being about 0.5 - 1.0 mg/ml. Currently preferred  
plasmids are those such as pGL2 (Promega), pSV40 $\beta$ -gal,  
pAd.CMVlacZ, and pLJ.

35

- 103 -

## EXAMPLE III

PARATHYROID HORMONE GENE CONSTRUCTS

5 The active fragment of the human parathyroid hormone gene (hPTH1-34) was chosen as the first of the osteotropic genes to be incorporated into an expression vector for use in gene transfer to promote new bone formation in the rat osteotomy model.

10 The inventors chose to construct the hPTH1-34 transgene in the pLJ expression vector (FIG. 10), since this vector was appropriate for studies of transgene function both in vitro and in vivo. A schematic of the PLJ-hPTH1-34 transgene is shown in FIG. 10. The DNA and  
15 amino acid sequences of the hPTH1-34 are well known, e.g., see Hendy et al., (1981, incorporated herein by reference). To insert the transgene into the PLJ expression vector PCR™ of a full-length PTH recombinant clone was employed, followed by standard molecular  
20 biological manipulation.

A retroviral stock was then generated following CaPO<sub>4</sub>-mediated transfection of  $\phi$  crip cells with the PLJ-hPTH1-34 construct, all according to standard protocols  
25 (Sambrook et al., 1989). Independent transduced Rat-1 clones were obtained by standard infection and selection procedures (Sambrook et al., 1989).

One clone (YZ-15) was analyzed by Southern analysis, demonstrating that the PLJ-hPTH1-34 transgene had stably  
30 integrated into the Rat-1 genome (FIG. 11). A Northern analysis was next performed to show that the YZ-15 clone expressed the PLJ-hPTH1-34 transgene, as evidenced by the presence of specific PLJ-hPTH1-34 transcripts (FIG. 12).

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- 104 -

## EXAMPLE IV

PARATHYROID HORMONE POLYPEPTIDE EXPRESSION AND ACTIVITY

A sensitive and specific radioimmunoassay was performed to demonstrate that the YZ-15 cells expressed and secreted a recombinant hPTH1-34 molecule (Table 2). The radioimmunoassay was performed on media from transduced Rat-1 clones. To quantify secretion of the recombinant hPTH-1-34 peptide produced by YZ-15 cells, the culture medium from one 100 mm confluent dish was collected over a 24 hour period and assayed with the NH<sub>2</sub>-terminal hPTH RIA kit (Nichols Institute Diagnostics) according to the manufacturer's protocol. PLJ-hPTH1-87 cells and BAG cells served as positive and negative controls, respectively.

Protein concentrations in Table 2 are expressed as the average of three assays plus the standard deviation (in parenthesis). The concentration of the 1-34 and full length (1-84) peptides was determined relative to a standard curve generated with commercially available reagents (Nichols Institute Diagnostics).

Table 2

CELL LINES	PTH (pg/ml)
YZ-15	247 (± 38)
PLJ-hPTH1-84	2616 (± 372)
BAG	13 (± 3)

As shown in Table 2, PTH expression was detected in both YZ-15 cells and PLJ-hPTH1-84 cells. BAG cells produced no detectable PTH and served as a baseline for the RIA. These results demonstrate that YZ-15 cells expressed recombinant hPTH1-34 protein.

The recombinant hPTH1-34 molecule was added to rat

- 105 -

osteosarcoma cells and a cAMP response assay conducted in order to determine whether the secreted molecule had biological activity. Unconcentrated media was collected from YZ-15 cells, PLJ-hPTH1-84 cells, and BAG cells and was used to treat ROS17/2.8 cells for 10 minutes, as described (Majmudar et al., 1991). cAMP was then extracted from treated cells and quantified by RIA (Table 3). The amount of cAMP shown is the average of three assays. The standard deviation of the mean is shown in parenthesis.

Table 3

CELL LINES	cAMP (pmol)
YZ-15	20.3 ( $\pm$ 0.25)
PLJ-hPTH184	88.5 ( $\pm$ 4.50)
BAG	7.6 ( $\pm$ 0.30)

A cAMP response was induced by the recombinant PTH secreted by the YZ-15 cells and by PLJ-hPTH1-84 cells. BAG cells produced no PTH and served as the baseline for the cAMP assay. These results provide direct *in vitro* evidence that the PLJ-hPTH1-34 transgene directs the expression and secretion of a functional osteotropic agent.

#### EXAMPLE V

##### BONE MORPHOGENETIC PROTEIN (BMP) GENE CONSTRUCTS

The murine bone morphogenetic protein-4 (BMP-4) was chosen as the next of the osteotropic genes to be incorporated into an expression vector for use in promoting bone repair and regeneration.

A full length murine BMP-4 cDNA was generated by screening a murine 3T3 cell cDNA library (Stratagene). The human sequence for BMP-4 is well known to those of

- 106 -

skill in the art and has been deposited in Genbank. Degenerate oligonucleotide primers were prepared and employed in a standard PCR™ to obtain a murine cDNA sequence.

5

The ends of the cDNA clone were further modified using the polymerase chain reaction so that the full length cDNA (5'→3' direction) encodes the natural murine initiator Met codon, the full length murine coding sequence, a 9 amino acid tag (known as the HA epitope), and the natural murine stop codon. The amino acid sequence encoded by the murine BMP-4 transgene is shown in FIG. 24; this entire sequence, including the tag, is represented by SEQ ID NO:1.

15

Placement of the HA epitope at the extreme carboxy terminus should not interfere with the function of the recombinant molecule sequence *in vitro* or *in vivo*. The advantage of the epitope is for utilization in immunohistochemical methods to specifically identify the recombinant murine BMP-4 molecule in osteotomy tissues *in vivo*, e.g., the epitope can be identified using a commercially available monoclonal antibody (Boehringer-Mannheim), as described herein.

25

Studies to demonstrate that the murine BMP-4 transgene codes for a functional osteotropic agent include, for example, (a) transfection of COS cells and immunoprecipitation of a protein band of the correct size using a monoclonal anti-HA antibody (Boehringer-Mannheim); and (b) a quantitative *in vivo* bone induction bioassay (Sampath and Reddi, 1981) that involves implanting proteins from the medium of transfected COS cells beneath the skin of male rats and scoring for new bone formation in the ectopic site.

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- 107 -

## EXAMPLE VI

DETECTION OF mRNA BY TISSUE IN SITU HYBRIDIZATION

The following technique describes the detection of mRNA in tissue obtained from the site of bone regeneration. This may be useful for detecting expression of the transgene mRNA itself, and also in detecting expression of hormone or growth factor receptors or other molecules. This method may be used in place of, or in addition to, Northern analyses, such as those described in FIG. 13.

DNA from a plasmid containing the gene for which mRNA is to be detected is linearized, extracted, and precipitated with ethanol. Sense and antisense transcripts are generated from 1 mg template with T3 and T7 polymerases, e.g., in the presence of [<sup>35</sup>S] UTP at >6 mCi/ml (Amersham Corp., >1200 Ci/mmol) and 1.6 U/ml RNasin (Promega), with the remaining in vitro transcription reagents provided in a kit (SureSite, Novagen Inc.). After transcription at 37°C for 1 hour, DNA templates are removed by a 15 minute digestion at 37°C with 0.5 U/ml RNase-free DNase I, extracted, and precipitated with ethanol. Riboprobes are hydrolyzed to an average final length of 150 bp by incubating in 40 mM NaHCO<sub>3</sub>, 60 mM Na<sub>2</sub>CO<sub>3</sub>, 80 mM DTT at 60°C, according to previously determined formula. Hydrolysis is terminated by addition of sodium acetate, pH 6.0, and glacial acetic acid to 0.09 M and 0.005% (v/v), respectively, and the probes are then ethanol precipitated, dissolved in 0.1 M DTT, counted, and stored at -20°C until use.

RNase precautions are taken in all stages of slide preparation. Bouins fixed, paraffin embedded tissue sections are heated to 65°C for 10 minutes, deparaffinized in 3 changes of xylene for 5 minutes, and rehydrated in a descending ethanol series, ending in

- 108 -

phosphate-buffered saline (PBS). Slides will be soaked in 0.2 N HCl for 5 min., rinsed in PBS, digested with 0.0002% proteinase K in PBS for 30 minutes at 37°C and rinsed briefly with DEPC-treated water. After

5 equilibrating for 3 minutes in 0.1 M triethanolamine-HCl (TEA-HCl), pH 8.0, sections are acetylated in 0.25% (v/v) acetic anhydride in 0.1 M TEA-HCl for 10 minutes at room temperature, rinsed in PBS, and dehydrated in an ascending ethanol series. Each section receives 100-200

10 ml prehybridization solution (0.5 mg/ml denatured RNase-free tRNA (Boehringer-Mannheim), 10 mM DTT, 5 mg/ml denatured, sulfurylated salmon sperm DNA, 50% formamide, 10% dextran sulfate, 300 mM NaCl, 1X RNase-free Denhardt's solution (made with RNase-free bovine serum

15 albumin, Sigma), 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and then incubate on a 50°C slide warmer in a humidified enclosure for 2 hours. The sulfurylated salmon-sperm DNA blocking reagent is used in both prehybridization and hybridization solutions to help reduce nonspecific

20 binding to tissue by <sup>35</sup>S groups on the probe. It is prepared by labeling RNase-free salmon sperm DNA (Sigma) with non-radioactive  $\alpha$ -thio-dCTP and  $\alpha$ -thio-dATP (Amersham) in a standard random oligonucleotide-primed DNA labeling reaction. Excess prehybridization solution

25 is removed with a brief rinse in 4X SSC before application of probe.

Riboprobes, fresh tRNA and sulfurylated salmon sperm DNA will be denatured for 10 minutes at 70°C, and chilled

30 on ice. Hybridization solution, identical to prehybridization solution except with denatured probe added to  $5 \times 10^6$  CPM/ml, is applied and slides incubated at 50°C overnight in sealed humidified chambers on a slide warmer. Sense and antisense probes are applied to

35 serial sections. Slides are rinsed 3 times in 4X SSC, washed with 2X SSC, 1 mM DTT for 30 min. at 50°C, digested with RNase A (20 mg/ml RNase A, 0.5 M NaCl, 10

- 109 -

mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) for 30 min. at 37°C, and rinsed briefly with 2X SSC, 1 mM DTT. Three additional washes are performed, each at 50°C for 30 minutes: once in 2X SSC, 50% formamide, 1 mM DTT, and  
5 twice in 1X SSC, 0.13% (w/v) sodium pyrophosphate, 1 mM DTT.

Slides are dehydrated in an ascending ethanol series (with supplementation of the dilute ethanols (50% and  
10 70%) with SSC and DTT to 0.1X and 1 mM, respectively). Slides are exposed to X-ray film for 20-60 hours to visualize overall hybridization patterns, dipped in autoradiographic emulsion (Kodak NTB-2, diluted to 50% with 0.3 M ammonium acetate), slowly dried for 2 hours,  
15 and exposed (4°C) for periods ranging from 8 days to 8 weeks. After developing emulsion, sections are counterstained with hematoxylin and eosin, dehydrated, and mounted with xylene-based medium. The hybridization signal is visualized under darkfield microscopy.

20

The above *in situ* hybridization protocol may be used, for example, in detecting the temporal and spatial pattern of PTH/PTHrP receptor expression. A suitable rat PTH/PTHrP receptor cDNA probe (R15B) is one that consists  
25 of a 1810 bp region encoding the full length rat bone PTH/PTHrP receptor (Abou-Samra et al., 1992). The cDNA fragment is subcloned into pcDNA 1 (Invitrogen Corp., San Diego, CA) and is cut out using *Xba*I and *Bam*HI. This probe has provided positive signals for northern blot  
30 analysis of rat, murine, and human osteoblastic cell lines, rat primary calvarial cells, and murine bone tissue. The pcDNA I plasmid contains a T7 and SP6 promoter that facilitate the generation of cRNA probes for *in situ* hybridization. The full length transcript  
35 has been used to detect PTH/PTHrP receptor in sections of bone (Lee et al., 1994). The PTHrP cDNA probe (Yasuda et al., 1989) is a 400 bp subcloned fragment in

- 110 -

pBluescript (Stratagene). This probe has been used for *in situ* hybridization, generating an antisense cRNA probe using *Bam*HI cleavage and the T3 primer and a sense cRNA probe using *Eco*RI cleavage and the T7 primer.

5

#### EXAMPLE VII

#### IN VIVO PROTEIN DETECTION FOLLOWING TRANSGENE EXPRESSION

##### 1. $\beta$ -galactosidase Transgene

10

Bacterial  $\beta$ -galactosidase can be detected immunohistochemically. Osteotomy tissue specimens are fixed in Bouins fixative, demineralized, and then split in half along the longitudinal plane. One-half of each specimen is embedded in paraffin for subsequent immunohistochemical identification of the bacterial  $\beta$ -galactosidase protein.

For immunohistochemistry, cross-Sections (2-3 mm thick) were transferred to poly-L-Lysine coated microscope slides and fixed in acetone at 0°C for at least 20 min. Sections were rehydrated in PBS. Endogenous peroxidase activity was quenched by immersion of tissue sections in 0.1% hydrogen peroxide (in 95% methanol) at room temperature for 10 min, and quenched sections were washed 3x in PBS. In some cases, sectioned calvariae were demineralized by immersion in 4% EDTA, 5% polyvinyl pyrrolidone, and 7% sucrose, pH 7.4, for 24 h at 4°C. Demineralized sections were washed 3x before application for antibodies. Primary antibodies were used without dilution in the form of hybridoma supernatant. Purified antibodies were applied to tissue sections at a concentration of 5 mg/ml. Primary antibodies were detected with biotinylated rabbit antimouse IgG and peroxidase conjugated streptavidin (Zymed Histostain-SPkit). After peroxidase staining, sections were counterstained with hematoxylin.

- 111 -

Bacterial  $\beta$ -gal can also be detected by substrate utilization assays. This is conducted using commercially available kits (e.g., Promega) according to the manufacturers' instructions.

5

## 2. Luciferase Transgene

Luciferase can be detected by substrate utilization assays. This is conducted using commercially available kits (e.g., Promega) according to the manufacturers' instructions.

10

## 3. PTH Transgenes

15

Recombinant PTH, such as hPTH1-34 peptide, is assayed in homogenates of osteotomy gap tissue, for example, using two commercially available radioimmunoassay kits according to the manufacturer's protocols (Nichols Institute Diagnostics, San Juan Capistrano, CA).

20

One kit is the Intact PTH-Parathyroid Hormone 100T Kit. This radioimmunoassay utilizes an antibody to the carboxy terminus of the intact hormone, and thus is used to measure endogenous levels of hormone in gap osteotomy tissue. This assay may be used to establish a baseline value PTH expression in the rat osteotomy model.

25

The second kit is a two-site immunoradiometric kit for the measurement of rat PTH. This kit uses affinity purified antibodies specific for the amino terminus of the intact rat hormone (PTH1-34) and thus will measure endogenous PTH production as well as the recombinant protein. Previous studies have shown that these antibodies cross-react with human PTH and thus are able to recognize recombinant molecules in vivo.

30

35

- 112 -

Values obtained with kit #1 (antibody to the carboxy terminus) are subtracted from values obtained with kit #2 (antibody to the amino terminus) to obtain an accurate and sensitive measurements. The level of recombinant peptide is thus correlated with the degree of new bone formation.

#### 4. BMP Transgene

Preferably, BMP proteins, such as the murine BMP-4 transgene peptide product, are detected immunohistochemically using a specific antibody that recognizes the HA epitope (Majmudar et al., 1991), such as the monoclonal antibody available from Boehringer-Mannheim. Antibodies to BMP proteins themselves may also be used. Such antibodies, along with various immunoassay methods, are described in U.S. Patent 4,857,456, incorporated herein by reference.

Osteotomy tissue specimens are fixed in Bouins fixative, demineralized, and then split in half along the longitudinal plane. One-half of each specimen is embedded in paraffin for subsequent immunohistochemical identification of the recombinant murine BMP-4 molecule.

#### EXAMPLE VIII

##### DIRECT GENE TRANSFER INTO REGENERATING BONE IN VIVO

To assess the feasibility of direct gene transfer into regenerating bone in vivo, marker gene transfer into cells in the rat osteotomy model was employed. These studies involved two marker genes: bacterial  $\beta$ -galactosidase and insect luciferase.

Aliquots of a fibrous collagen implant material were soaked in solutions of pure marker gene DNA. The implant

- 113 -

materials were then placed in the osteotomy site, and their expression determined as described above.

It was found that both marker genes were  
5 successfully transferred and expressed, without any  
failures, as demonstrated by substrate utilization assays  
(FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C and FIG.  
6D). Since mammalian cells do not normally synthesize  
either marker gene product, this provides direct evidence  
10 that osteotomy repair cells were transfected *in vivo* and  
then expressed the  $\beta$ -galactosidase and luciferase  
transgenes as a functional enzymes.

#### EXAMPLE IX

##### 15 ADENOVIRAL GENE TRANSFER INTO REGENERATING BONE IN VIVO

One of the alternative methods to achieve *in vivo*  
gene transfer into regenerating bone is to utilize an  
adenovirus-mediated transfer event. Successful  
20 adenoviral gene transfer of a marker gene construct into  
bone repair cells in the rat osteotomy model has been  
achieved (FIG. 23A, FIG. 23B, and FIG. 23C).

The inventors employed the adenoviral vector pAd.  
25 CMVlacZ, which is an example of a replication-defective  
adenoviral vector which can replicate in permissive cells  
(Stratford-Perricaudet et al., 1992). In pAd.CMVlacZ,  
the early enhancer/promoter of the cytomegalovirus (CMV)  
is used to drive transcription of lacZ with an SV40  
30 polyadenylation sequence cloned downstream from this  
reporter (Davidson et al., 1993).

The vector pAd.RSV4 is also utilized by the  
inventors. This vector essentially has the same backbone  
35 as pAdCMVlacZ, however the CMV promoter and the single  
BglII cloning site have been replaced in a cassette-like  
fashion with BglII fragment that consists of an RSV

- 114 -

promoter, a multiple cloning site, and a poly(A<sup>+</sup>) site. The greater flexibility of this vector is contemplated to be useful in subcloning osteotropic genes, such as the hPTH1-34 cDNA fragment, for use in further studies.

5

To generate recombinant PTH adenovirus, a 100-mm dish of 293 cells is transfected using calcium phosphate with 20 mg of a plasmid construct, e.g., the plasmid containing the hPTH1-34 insert linearized with *NheI*, plus  
10 2 mg of wild type adenovirus DNA digested with *XbaI* and *ClaI*. The adenovirus DNA is derived from adenovirus type 5, which contains only a single *XbaI* and *ClaI* sites and has a partial deletion of the E3 region. Approximately 7 days post-transfection, cells and media are harvested and  
15 a lysate prepared by repeated freeze-thaw cycles. This lysate is diluted and used to infect 60 -mm dishes of confluent 293 cells for 1 hour. The cells are then overlaid with 0.8% agar/1X MEM/2% calf serum/12.5 mM  $MgCl_2$ . Ten days post-infection, individual plaques are to  
20 be picked and used to infect 60-mm dishes of 293 cells to expand the amount of virus. Positive plaques are selected for further purification and the generation of adenoviral stocks.

25 To purify recombinant adenovirus, 150-mm dishes of 75-90% confluent 293 cells are infected with 2-5 PFU/cell, a titer that avoids the potential cytotoxic effects of adenovirus. Thirty hours post-infection, the cells are rinsed, removed from the dishes, pelleted, and  
30 resuspended in 10 mM Tris-HCl, pH 8.1. A viral lysate is generated by three freeze-thaw cycles, cell debris is removed by centrifugation for 10 min. at 2,000 rpm, and the adenovirus is purified by density gradient centrifugation. The adenovirus band is stored at -20°C  
35 in sterile glycerol/BSA until needed.

The solution of virus particles was sterilized and



- 115 -

incubated with the implant material (from 6 min to overnight), and the virus-impregnated material was implanted into the osteotomy gap; where viral infection of cells clearly occurred. The results obtained clearly demonstrated the exquisite specificity of the anti- $\beta$ -gal antibody (Sambrook et al., 1989), and conclusively demonstrated expression of the marker gene product in chondrocyte-like cells of the osteotomy gap. The nuclear-targeted signal has also been observed in pre-osteoblasts.

#### EXAMPLE X

##### TRANSFER OF AN OSTEOTROPIC GENE STIMULATES BONE REGENERATION/REPAIR IN VIVO

15

In order for a parathyroid hormone (PTH) transgene to function as an osteotropic agent, it is likely that there is a requirement for the PTH/PTHrP receptor to be expressed in the bone repair tissue itself. Therefore, the inventors investigated PTH/PTHrP receptor expression in the rat osteotomy model.

A Northern analysis of poly-A(+) RNA was conducted which demonstrated that the PTH/PTHrP receptor was expression in osteotomy repair tissue (FIG. 13).

The inventors next investigated whether gene transfer could be employed to create transfected cells that constitutively express recombinant hPTH1-34 *in vivo*, and whether this transgene can stimulate bone formation. The rate of new bone formation is analyzed as follows. At necropsy the osteotomy site is carefully dissected for histomorphometric analysis. The A-P and M-L dimensions of the callus tissue are measured using calipers. Specimens are then immersion fixed in Bouins fixative, washed in ethanol, and demineralized in buffered formic acid. Plastic embedding of decalcified materials is used because of the superior dimensional stability of

- 116 -

methacrylate during sample preparation and sectioning.

Tissue blocks are dehydrated in increasing alcohol concentrations and embedded. 5 mm thick sections are cut in the coronal plane using a Reichert Polycut microtome. Sections are prepared from midway through the width of the marrow cavity to guard against a sampling bias. Sections for light microscopy are stained using a modified Goldner's trichrome stain, to differentiate bone, osteoid, cartilage, and fibrous tissue. Sections are cover-slipped using Eukitt's mounting medium (Calibrated Instruments, Ardsley, NY). Histomorphometric analyses are performed under brightfield using a Nikon Optiphot Research microscope. Standard point count stereology techniques using a 10 mm x 10 mm eyepiece grid reticular are used.

Total callus area is measured at 125X magnification as an index of the overall intensity of the healing reaction. Area fractions of bone, cartilage, and fibrous tissue are measured at 250 X magnification to examine the relative contribution of each tissue to callus formation. Since the dimensions of the osteotomy gap reflect the baseline (time 0), a measurement of bone area at subsequent time intervals is used to indicate the rate of bone infill. Statistical significance is assessed using analysis of variance, with post-hoc comparisons between groups conducted using Tukey's studentized range t test.

In the 5-mm rat osteotomy model described above, it was found that PTH transgene expression can stimulate bone regeneration/repair in live animals (FIG. 6A, FIG. 6B, FIG. 6C, and FIG. 6D). This is a particularly important finding as it is known that hPTH1-34 is a more powerful anabolic agent when given intermittently as opposed to continuously, and it is the continuous-type delivery that results from the gene transfer methods used

here.

Although the present inventors have already demonstrated success of direct gene transfer into regenerating bone *in vivo*, the use of *ex vivo* treatment protocols is also contemplated. In such embodiments, bone progenitor cells would be isolated from a particular animal or human subject and maintained in an *in vitro* environment. Suitable areas of the body from which to obtain bone progenitor cells are areas such as the bone tissue and fluid surrounding a fracture or other skeletal defect (whether or not this is an artificially created site) and from the bone marrow. Isolated cells would then be contacted with the DNA (or recombinant viral) composition, with, or preferably without, a matrix, when the cells would take up the DNA (or be infected by the recombinant virus). The stimulated cells would then be returned to the site in the animal or patient where bone repair is to be stimulated.

20

#### EXAMPLE XI

##### **TRANSFER OF GENES TO ACHILLES' TENDON AND TO CRUCIATE LIGAMENT *IN VIVO***

The studies on regenerating bone described above complement others by the inventors in which gene transfer was successfully employed to introduce genes into Achilles' tendon (FIG 3A, FIG. 3B, FIG. 3C, FIG. 3D, and FIG. 3E) and cruciate ligament (FIG. 4).

30

The Achilles' tendon consist of cells and extracellular matrix organized in a characteristic tissue architecture. Tissue wounding can disrupt this architecture and stimulate a wound healing response. The wounded tendon will regenerate, as opposed to scar, if its connective tissue elements remain approximately intact. Regeneration is advantageous because scar tissue is not optimally designed to support normal mechanical

function. Segmental defects in tendon due to traumatic injury may be treated with biological or synthetic implants that encourage neo-tendon formation. This strategy is limited, however, by the availability of effective (autologous) biological grafts, the long term stability and compatibility of synthetic prostheses, and the slow rate of incorporation often observed with both types of implants.

10       The inventors hypothesized that the effectiveness of biological grafts may be enhanced by the over-expression of molecules that regulate the tissue regeneration response. Toward this end, they developed a model system in which segmental defects in Achilles' tendon are  
15       created and a novel biomaterial, is used as a tendon implant/molecular delivery agent. In the present example, the ability to deliver and express marker gene constructs into regenerating tendon tissue is demonstrated.

20       Plasmid (pSV $\beta$ gal, Promega) stock solutions were prepared according to standard protocols (Sambrook et al., 1989). SIS graft material was prepared from a segment of jejunum of adult pigs (Badylak et al., 1989).  
25       At harvest, mesenteric tissues were removed, the segment was inverted, and the mucosa and superficial submucosa were removed by a mechanical abrasion technique. After returning the segment to its original orientation, the serosa and muscle layers were rinsed, sterilized by  
30       treatment with dilute peracetic acid, and stored at 4°C until use.

      Mongrel dogs (all studies) were anesthetized, intubated, placed in right-lateral recumbency upon a  
35       heating pad, and maintained with inhalant anesthesia. A lateral incision from the musculotendinous junction to the plantar fascia was used to expose the Achilles'

- 119 -

tendon. A double thickness sheet of SIS was wrapped around a central portion of the tendon, both ends were sutured, a 1.5 cm segment of the tendon was removed through a lateral opening in the graft material, and the graft and surgical site were closed. The leg was immobilized for 6 weeks and then used freely for 6 weeks. Graft tissues were harvested at time points indicated below, fixed in Bouins solution, and embedded in paraffin. Tissue sections (8  $\mu$ m) were cut and used for immunohistochemistry.

In an initial study, SIS material alone (SIS-alone graft) engrafted and promoted the regeneration of Achilles' tendon following the creation of a segmental defect in mongrel dogs as long as 6 months post surgery. The remodeling process involved the rapid formation of granulation tissue and eventual degradation of the graft. Scar tissue did not form, and evidence of immune-mediated rejection was not observed.

In a second study, SIS was soaked in a plasmid DNA solution (SIS+plasmid graft) and subsequently implanted as an Achilles' tendon graft (n=2 dogs) or a cruciate ligament graft (n=2 dogs) in normal mongrel dogs. A pSV $\beta$ gal plasmid that employs simian virus 40 regulatory sequences to drive  $\beta$ -galactosidase ( $\beta$ -gal) activity was detectable by immunohistochemistry using a specific antibody in 4/4 animals. As a negative control,  $\beta$ -gal activity was not detected in the unoperated Achilles' tendon and cruciate ligament of these animals. It appeared, therefore, that SIS facilitated the uptake and subsequent expression of plasmid DNA by wound healing cells in both tendon and ligament.

A third study was designed to evaluate the time course of  $\beta$ -gal transgene expression. SIS + plasmid grafts were implanted for 3, 6, 9, and 12 weeks (n=2 dogs

- 120 -

pr time point) and transgene expression was assayed by immunohistochemistry and by *in situ* hybridization. Cross-sections (8- $\mu$ m) of Bouins fixed, paraffin embedded tissue were cut and mounted on ProbeOn Plus slides (Fisher). Immunohistochemistry was performed according to the protocol provided with the Histostain-SP kit (Zymed). In brief, slides were incubated with a well characterized anti- $\beta$ -galactosidase antibody (1:200 dilution, 5'→3'), washed in PBS, incubated with a biotinylated second antibody, washed, stained with the enzyme conjugate plus a substrate-chromogen mixture, and then counterstained with hematoxylin and eosin.

Bacterial  $\beta$ -gal activity was detected in tendons that received the SIS+plasmid graft (8/8 animals). Although not rigorously quantitative, transgene expression appeared to peak at 9-12 weeks. Bacterial  $\beta$ -gal gene expression was not detected in animals that received SIS-alone grafts (N=2, 3 weeks and 12 weeks). Again, scar tissue did not form and evidence of immune-mediated rejection was not observed.

This study demonstrated that the mucosal biomaterial SIS can function as an autologous graft that promotes the regeneration of tissues such as Achilles' tendon and anterior cruciate ligament. SIS can also be used to deliver a marker gene construct to regenerating tissue.

#### EXAMPLE XIII

##### 30      MECHANICAL PROPERTIES OF NEW BONE FORMATION

The mechanical properties of new bone formed during gene transfer may be measured using, e.g., whole bone torsion tests which create a stress state in which the maximum tensile stresses will occur on planes that lie obliquely to the bone's longitudinal axis. Such tests may provide important inferences about the mechanical

- 121 -

anisotropy of callus tissue and the degree of osseous integration of new bone tissue. These tests are particularly advantageous in the evaluation of fracture specimens, e.g., the irregular shape of callus tissue typically precludes the use of whole bone 4-point bending tests because it is impossible to reproducibly align the points from specimen to specimen.

Femurs are tested on an MTS Servohydraulic Testing Machine while moist and at room temperature. A torque sensor and rotary variable displacement transducer provides data for torque-angular displacement curves. Specially designed fixtures support each bone near the metaphyseal-diaphyseal junctions, and apply a 2-point load to the diaphysis. Tests are conducted at a constant rate of displacement equal to 20 degrees/sec. A 250 inch-ounce load cell measures the total applied force. All bones are tested while moist and room temperature. Torque and angular displacement data are acquired using an analog-to-digital converter and a Macintosh computer and software. From this data, the following variables are calculated: a) maximum torque, b) torsional stiffness, the slope of the pre-yield portion of the curve determined from a linear regression of the data, c) energy to failure, the area under the torque-angular displacement curve to the point of failure, and d) the angular displacement ratio, the ratio of displacement at failure to displacement at yield. Statistical significance is determined Analysis of Variance followed by multiple comparisons with appropriate corrections (e.g., Bonferroni).

This invention also provides a means of using osteotropic gene transfer in connection with reconstructive surgery and various bone remodelling procedures. The techniques described herein may thus be employed in connection with the technology described by

- 122 -

Yasko et al., 1992; Chen et al., 1991; and Beck et al., 1991, each incorporated herein by reference.

#### EXAMPLE XIV

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#### TYPE II COLLAGEN PROMOTES NEW BONE GROWTH

Certain matrix materials are capable of stimulating at least some new growth in their own right, i.e., are "osteoconductive materials". Potential examples of such materials are well known in the field of orthopedic research and include preparations of hydroxyapatite; preparations of crushed bone and mineralized collagen; PLGA block copolymers and polyanhydride. The ability of these materials to stimulate new bone formation distinguishes them from inert implant materials such as methylcellulose, which have in the past been used to deliver BMPs to sites of fracture repair.

This Example relates to a study using the rat osteotomy model with implants made of collagen type I (Sigma), collagen type II (Sigma), and UltraFiber™ (Norian Corp.). These materials have been placed *in situ* without DNA of any type. Five animals received an osteotomy with 10 mg of a type II collagen implant alone (10 mg refers to the original quantity of lyophilized collagen). Five of five control animals received an osteotomy with 10 mg of a type I collagen implant alone. Animals were housed for three weeks after surgery and then sacrificed.

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The results of these studies were that SIS appeared to retard new bone formation; type I collagen incited a moderately intense inflammatory response; and UltraFiber™ acted as an osteoconductive agent. The type II collagen implant studies yielded surprising results in that 10 mg of this collagen was found to promote new bone formation in the 5-mm osteotomy model (FIG. 22A, FIG. 22B, and FIG.



- 123 -

22C). New bone - bridging the osteotomy gap - was identified three weeks after surgery in 5/5 animals that received a type II collagen implant alone (i.e., minus DNA of any type). In contrast, fibrous granulation tissue, but no evidence of new bone formation, was obtained in 5/5 animals receiving a type I collagen implant alone.

Radiographic analysis demonstrated conclusively that all animals receiving an osteotomy with a type II collagen implant without exception showed radio-dense material in the osteotomy gap (FIG. 22A). In sharp contrast, radiographic analysis of all animals receiving a type I collagen implant revealed no radio-dense material forming in the osteotomy gap (FIG. 22B). The arrow in FIG. 22A point to the new bone growth formed in the osteotomy gap of type II collagen implanted-animals. No such new bone growth was observed in the animals receiving type I collagen implants (FIG. 22B).

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FIG. 22C demonstrates the results of the osteotomy with a type II collagen implant. The arrow points to the area of new bone formed in the osteotomy gap. In contrast, only fibrous granulation tissue was identified in the type I collagen gap.

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Previous studies have suggested that type II collagen plays only a structural role in the extracellular matrix. The results of the type II collagen implant studies are interesting because they demonstrate a novel and osteoconductive role for type II collagen during endochondral bone repair. To further optimize the osteoconductive potential of type II collagen, a yeast expression vector that encodes for type II collagen (full length  $\alpha 1(\text{II})$  collagen) will be employed to produce recombinant  $\alpha 1(\text{II})$  collagen protein.

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## EXAMPLE XV

## IDENTIFICATION OF FURTHER OSTEOTROPIC GENES:

ISOLATION OF A NOVEL LATENT TGF- $\beta$   
BINDING PROTEIN-LIKE (LTBP-3) GENE

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The TGF- $\beta$ s represent a family of structurally related molecules with diverse effects on mammalian cell shape, growth, and differentiation (Roberts and Sporn, 1990). Initially synthesized as a precursor consisting of an amino-terminal propeptide followed by mature TGF- $\beta$ , two chains of nascent pro-TGF- $\beta$  associate in most tissues to form a Mr ~106,000 inactive disulfide-bonded dimer. Homodimers are most common, but heterodimers have also been described (Cheifetz et al., 1987; Ogawa et al., 1992). During biosynthesis the mature TGF- $\beta$  dimer is cleaved from the propeptide dimer. TGF- $\beta$  latency results in part from the non-covalent association of propeptide and mature TGF- $\beta$  dimers (Pircher et al., 1984 and 1986; Wakefield et al., 1987; Millan et al., 1992; see also Miyazono and Heldin, 1989). Consequently, the propeptide dimer is often referred to as the latency associated protein (LAP), and LAP plus the disulfide-bonded TGF- $\beta$  dimer are also known as the small latent complex. In the extracellular space small latent complexes must be dissociated to activate mature TGF- $\beta$ . The mechanism of activation of the latent complex is thought to be one of the most important steps governing TGF- $\beta$  effects (Lyons et al., 1988; Antonelli-Orlidge et al., 1989; Twardzik et al., 1990; Sato et al., 1993).

In certain lines of cultured cells small latent growth factor complexes may contain additional high molecular weight proteins. The best characterized of these high molecular weight proteins is the latent TGF- $\beta$  binding protein, or LTBP (Miyazono et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990; Olofsson et al., 1992; Taketazu et al., 1994). LTBP produced by different cell

- 125 -

types is heterogeneous in size, perhaps because of alternative splicing or because of tissue-specific proteolytic processing (Miyazono et al., 1988; Wakefield et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990).

5 Latent TGF- $\beta$  complexes that contain LTBP are known as large latent complexes. LTBP has no known covalent linkage to mature TGF- $\beta$ , but rather it is linked by a disulfide bond to LAP.

10 Two LTBPs have been isolated to date. The deduced human LTBP-1 amino acid sequence is comprised of a signal peptide, 16 epidermal growth factor-like repeats with the potential to bind calcium (EGF-CB repeats), 2 copies of a unique motif containing 8 cysteine residues, an RGD cell  
15 attachment motif, and an 8 amino acid motif identical to the cell binding domain of the laminin B2 chain (Kanzaki et al., 1990). There is evidence that LTBP-1 binds calcium, which, in turn, induces a structural change that protects LTBP from proteolytic attack (Colosetti et al.,  
20 1993). LTBP-2 shows 41% sequence identity to LTBP-1, and its structural domains show a similar overall organization (Moren et al., 1994).

While the functions of LTBP-1 and LTBP-2 presently  
25 are unknown, several ideas have been put forward in the literature. First, LTBP may regulate the intracellular biosynthesis of latent TGF- $\beta$  precursors. Cultured erythroleukemia cells efficiently assemble and secrete large latent TGF- $\beta$  complexes, whereas they slowly secrete  
30 small latent TGF- $\beta$  complexes that contain anomalous disulfide bonds (Miyazono et al., 1991; Miyazono et al., 1992). Therefore, LTBP may facilitate the normal assembly and secretion of latent TGF- $\beta$  complexes. Second, LTBP may target latent TGF- $\beta$  to specific types of  
35 connective tissue. Recent evidence suggests that the large latent TGF- $\beta$  complex is covalently bound to the extracellular matrix via LTBP (Taipale et al., 1994).

- 126 -

Based on these observations, LTBP has been referred to as a "matrix receptor", i.e. a secreted protein that targets and stores latent growth factors such as TGF- $\beta$  to the extracellular matrix. Third, LTBP may modulate the activation of latent complexes. This idea is based in part on recent evidence which suggests that mature TGF- $\beta$  is released from extracellular storage sites by proteases such as plasmin and thrombin and that LTBP may protect small latent complexes from proteolytic attack (Falcone et al., 1993; Benezra et al., 1993; Taipale et al., 1994), i.e. protease activity may govern the effect of TGF- $\beta$  in tissues, but LTBP may modulate this activity. Fourth, LTBP may play an important role in targeting the latent TGF- $\beta$  complex to the cell surface, allowing latent TGF- $\beta$  to be efficiently activated (Flaumenhaft et al., 1993).

#### A. MATERIALS AND METHODS

##### 1. cDNA Cloning

Aliquots (typically 40-50,000 PFU) of phage particles from a cDNA library in the  $\lambda$ ZAPII<sup>®</sup> vector made from NIH 3T3 cell mRNA (Stratagene) and fresh overnight XL1-Blue<sup>™</sup> cells (grown in Luria broth supplemented with 0.4% maltose in 10 mM MgSO<sub>4</sub>) were mixed, incubated for 15 min. at 37°C, mixed again with 9 ml of liquid (50°C) top layer agarose (NZY broth plus 0.75% agarose), and then spread evenly onto freshly poured 150 mm NZY-agar plates. Standard methods were used for the preparation of plaque-lifts and filter hybridization (42°C, in buffer containing 50% formamide, 5X SSPE, 1X Denhardt's, 0.1% SDS, 100 mg/ml salmon sperm DNA, 100 mg/ml heparin). Filters were washed progressively to high stringency (0.1X SSC/0.1% SDS, 65°C). cDNA probes were radiolabeled by the nick translation method using commercially available reagents and protocols (Nick Translation Kit,

- 127 -

Boehringer Mannheim). Purified phage clones were converted to pBluescript® plasmid clones, which were sequenced using Sequenase (v2.0) as described (Chen et al., 1993; Yin et al., 1995). Sequence alignment and identity was determined using sequence analysis programs from the Genetics Computer Group (MacVector).

## 2. Tissue In Situ Hybridization

To prepare normal sense and antisense probes, a unique 342 bp fragment from the 3' untranslated region (+3973 to +4314, counting the "A" of the initiator Met codon as +1; see "ish", Fig. 1) was subcloned into the pBSKS+ plasmid (Stratagene, Inc.). Template DNA was linearized with either EcoRI or BamHI, extracted, and precipitated with ethanol. Sense and antisense transcripts were generated from 1 mg template with T3 and T7 polymerases in the presence of [<sup>35</sup>S]UTP at >6 mCi/ml (Amersham, >1200 Ci/mmol) and 1.6 U/ml RNasin (Promega), with the remaining *in vitro* transcription reagents provided in a kit (SureSite, Novagen Inc.). After transcription at 37°C for 1 h, DNA templates were removed by a 15 min. digest at 37°C with 0.5 U/ml RNase-free DNase I, extracted, and precipitated with ethanol. Riboprobes were hydrolyzed to an average final length of 150 bp by incubating in 40 mM NaHCO<sub>3</sub>, 60 mM Na<sub>2</sub>CO<sub>3</sub>, 80 mM DTT for ~40 min. at 60°C. Hydrolysis was terminated by addition of sodium acetate, pH 6.0, and glacial acetic acid to .09 M and 0.56% (v/v), respectively, and the probes were then ethanol precipitated, dissolved in 0.1 M DTT, counted, and stored at -20°C until use. Day 8.5-9.0, day 13.5, and day 16.5 mouse embryo tissue sections (Novagen) and the *in situ* hybridization protocol

were exactly as described (Chen et al., 1993; Yin et al., 1995).

### 3. Northern Analysis

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MC3T3-E1 cell poly(A+) RNA (2-10 mg aliquots) was electrophoresed on a 1.25% agarose/2.2 M formaldehyde gel and then transferred to a nylon membrane (Hybond-N, Amersham). The RNA was cross-linked to the membrane by exposure to a UV light source ( $1.2 \times 10^6$  mJ/cm<sup>2</sup>, UV Stratalinker 2400, Stratagene) and then pre-hybridized for >15 min. at 65°C in Rapid-Hyb buffer (Amersham, Inc.). A specific cDNA probe consisting solely of untranslated sequence from the 3' end of the transcript was <sup>32</sup>P-labeled by random priming and used for hybridization (2 h at 65°C). Blots were washed progressively to high stringency (0.1X SSC/0.1% SDS, 65°C), and then placed against x-ray film with intensifying screens (XAR, Kodak) at -86°C.

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### 4. Antibody Preparation

LTBP-3 antibodies were raised against a unique peptide sequence found in domain #2 (amino acids 155-167). Peptide #274 (GESVASKHAIYAVC) (SEQ ID NO:16) was synthesized using an ABI model 431A synthesizer employing FastMoc chemistry. The sequence was confirmed using an ABI473 protein sequencer. A cysteine residue was added to the carboxy-terminus to facilitate crosslinking to carrier proteins. For antibody production, the synthetic peptide was coupled to rabbit serum albumin (RSA) using MBS (*m*-maleimidobenzoic acid-*N*-hydroxysuccinimide ester) at a substitution of 7.5 mg peptide per mg of RSA. One mg of the peptide-RSA conjugate in 1 ml of Freund's complete adjuvant was injected subcutaneously at 10 different sites along the backs of rabbits. Beginning at 3 weeks after initial

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- 129 -

immunization, the rabbits were given bi-weekly booster injections of 1 mg peptide-RSA in 100 ul of Freund's incomplete adjuvant. IgG was prepared by mixing immune serum with caprylic acid (0.7 ml caprylic acid per ml serum), stirring for 30 min., and centrifuging at 5,000 x g for 10 min. The supernatant was decanted and dialyzed against two changes of phosphate buffered saline (PBS) overnight at 4°C. The antibody solution was then affinity purified by passing it over a column containing the immunizing peptide coupled to Affi-gel 10 affinity support. Bound antibodies were eluted with 0.2 M glycine (pH 2.3), immediately dialyzed against PBS, and concentrated to 1 mg/ml. prior to storage at -70°C.

## 5. Transfection

Transient transfection was performed using standard protocols (Sambrook et al., 1989). Briefly, subconfluent cells (covering ~20% of a 100 mm plastic tissue culture dish) were washed 2x in DMEM tissue culture medium (GIBCO) and then incubated for 3 hrs. at 37°C in a sterile mixture of DEAE-dextran (0.25 mg/ml), chloroquine (55 mg/ml), and 15 mg plasmid DNA (Courey and Tjian, 1988). Cells then were shocked by incubation with 10% DMSO in sterile PBS for 2 min. at 37°C, washed 2x with DMEM (Sambrook et al., 1989), and incubated in DMEM plus 10% fetal calf serum and antibiotics for 72 hr. at 37°C.

## 6. Immunoprecipitation

For immunoprecipitation, 1 ml of antibody (1:400 final concentration, in PBS-TDS buffer: 0.38 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 1% Triton X-100, 0.5% Deoxycholic acid, and 0.1% SDS) was added to 1 ml of radiolabeled medium proteins. The mixture was incubated with shaking at 4°C for 1 hr., protein A-sepharose CL-4B beads were added (200 ml, 10% suspension), and this

- 130 -

5 mixture was incubated with shaking for one additional  
hour at 4°C. Immunoprecipitated proteins were pelleted  
by brief centrifugation, the pellet was washed 6x with  
PBS-TDS buffer, 2x protein loading dye was added, and the  
10 samples were boiled for 5 min. and then fractionated on  
4-18% gradient SDS-PAGE (Bonadio et al., 1985). Cold  
molecular weight markers (200 kDa-14.3 kDa, Rainbow mix,  
Amersham) were used to estimate molecular weight. The  
gel was dried and exposed to film for the indicated time  
at room temperature.

### 7. Western Analysis

15 Fractionated proteins within SDS-polyacrylamide gels  
were transferred to a nitrocellulose filter for 2 hours  
using Tris-glycine-methanol buffer, pH 8.3 at 0.5 mA/cm<sup>2</sup>.  
The filter was blocked, incubated with nonfat milk plus  
antibody (1:1000 dilution) for 2 hr, and washed.  
Antibody staining was visualized using the ECL Western  
20 blotting reagent (Amersham) according to the  
manufacturer's protocols.

### B. RESULTS

25 In this study, the inventors isolated and  
characterized a novel murine fibrillin-like cDNA encoding  
LTBP-3. To clone the murine LTBP-3 gene, cDNA from a 3T3  
cell cDNA library was amplified using human fibrillin-1  
PCR™ primers under low stringency conditions (i.e.,  
30 annealing at 37°C initially for 10 cycles, followed by  
annealing at 60°C for 30 cycles). The results indicated  
that a murine DNA fragment of unexpectedly low homology  
(~50%) to human fibrillin-1 was obtained. Molecular  
cloning of the authentic murine fibrillin-1 transcript  
35 was also performed, confirming the human and murine  
fibrillin-1 coding sequences share >95% sequence  
identity. The murine fibrillin-1 and PCR™ sequences were



- 131 -

different, which suggested that the PCR™ product may have been derived from a related, fibrillin-like cDNA. The 3T3 cell cDNA library was screened at high stringency using the murine PCR™ product as the probe in order to test this hypothesis. A cDNA walking strategy eventually yielded seven overlapping cDNA clones (FIG. 14). It provides a unique mRNA of 4,314 nucleotides, with an open reading frame of 3,753 nucleotides (SEQ ID NO:2). The deduced molecule is a unique polypeptide of 1,251 amino acids (SEQ ID NO:3). Excluding the signal peptide (21 amino acids), the novel fibrillin-like molecule consists of five structurally distinct regions (Region 1- Region 5), and although similar to murine fibrillin-1 (FIG. 15A), its domain structure is unique as is evidenced by the schematic representation of LTBP-3 shown in FIG. 15B.

Domain #1 is a 28 amino acid segment with a net basic charge (est. pI, 12.36) that may allow for binding acidic molecules in the extracellular matrix (e.g., acidic proteoglycans). Sequences rich in basic amino acids may also function as endoproteolytic processing signals (Barr, 1991; Steiner et al., 1992), which suggests that the NH<sub>2</sub>-terminus may be proteolytically processed. Domain #2 extends for of 390 amino acids, consisting of an EGF-like repeat, a 135 amino acid segment that was proline-rich (20.7%) and glycine-rich (11.8%) but not cysteine-rich, a Fibmotif (Pereira et al., 1993), an EGF-CB repeat, and a TGF-bp repeat. Domain #3 is a 113 amino acid segment characterized by its high proline content (21%). Domain #4 extends for 678 amino acids and consists of 14 consecutive cysteine-rich repeats. Based on structural homologies, 12/14 repeats were epidermal growth factor-calcium binding (EGF-CB) motifs (Handford et al., 1991), whereas 2/14 were transforming growth factor- $\beta$ -binding protein (TGF-bp) motifs (Kanzaki et al., 1990). Finally, domain #5 is a 22 amino acid segment at the carboxy-terminus. The

- 132 -

conceptual amino acid sequence encoded by the open reading frame consisted of 1,251 amino acids (FIG. 15B) with an estimated pI of 5.92, a predicted molecular mass of 134,710 Da, and five potential N-linked glycosylation sites. No RGD sequence was present.

Northern blot analysis of murine embryo RNA using a 3' untranslated region probe identified a transcript band of ~4.6 kb. In this regard, 4,310 nt have been isolated by cDNA cloning, including a 3' untranslated region of 401 nt and a 5' upstream sequence of 156 nt. The apparent discrepancy between the Northern analysis result and the cDNA sequence analysis suggested that the 5' upstream sequence may include ~300 nt of additional upstream sequence. This estimate was consistent with preliminary primer extension mapping studies indicating that the 5' upstream sequence is 400-500 nt in length.

A total of 19 cysteine-rich repeats were found in domains #2 and #4 of the murine LTBP-like (LTBP-3) polypeptide. Thirteen were EGF-like and 11/13 contained the calcium binding consensus sequence. This consensus was derived from an analysis of 154 EGF-CB repeats in 23 different proteins and from structural analyses of the EGF-CB repeat, both bound and unbound to calcium ion (Selander-Sunnerhagen et al., 1992). Variations on the consensus have been noted previously and one of these, D-L-N/D-E-C<sub>1</sub>, was identified in the third EGF-like repeat of domain #4. In addition, a potential calcium binding sequence which has not previously been reported (E-T-N/D-E-C<sub>1</sub>) was identified in the first EGF-like repeat of domain #4. Ten of thirteen EGF-CB repeats also contained a second consensus sequence which represents a recognition sequence for an Asp/Asn hydroxylase that co- and post-translationally modifies D/N residues (Stenflo et al., 1987; Gronke et al., 1989).

- 133 -

Although about one-half the size, the deduced polypeptide was organized like fibrillin-1 in that it consisted of a signal peptide followed by 5 structurally distinct domains, *i.e.*, two domains with numerous EGF-like, EGF-CB and Fib repeats and a third with a proline-rich sequence (Pereira *et al.*, 1993). However, comparison of each of these domains using the GAP and BESTFIT programs (Genetics Computer Group) has revealed a low level of amino acid homology of only 27% over the five structural domains shared by the deduced murine polypeptide and human fibrillin-2. These values are low for a putative fibrillin family member because fibrillin-1 and fibrillin-2 share ~50% identity (Zhang *et al.*, 1994).

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A search of available databases revealed that the deduced murine polypeptide was most similar to the human and rat latent TGF- $\beta$  binding proteins (Kanzaki *et al.*, 1990; Tsuji *et al.*, 1990). In this regard LTBP was found to be similar to fibrillin in that it could also be divided into five structurally distinct domains (FIG. 15A, FIG. 15B, and FIG. 15C). These include a relatively short domain downstream of the signal peptide with a net basic charge (amino acids 21-33, *est.* pI, 11.14); a domain consisting of EGF-like, EGF-CB, TGF-bp, and Fib motifs plus a proline-rich and glycine-rich sequence (amino acids 34-407); a proline-rich domain (amino acids 408-545); a large, domain consisting of EGF-CB, TGF-bp, and TGF-bp-like repeat motifs (amino acids 546-1379); and a relatively short domain at the carboxy terminus (amino acids 1380-1394). Amino acid sequence comparison of the deduced murine and human polypeptides shows 60% identity for domain #1, 52% identity for domain #2, 30% identity for domain #3, 43% identity for domain #4, and 7% identity for domain #5. The average identity over the five domains shared by the murine polypeptide and human

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- 134 -

LTBP was 38.4%. Significantly, cysteine residues in both polypeptide sequences were highly conserved.

The fibrillins are exclusively expressed by  
5 connective cells in developing tissues (Zhang et al.,  
1994), whereas LTBP should be expressed along with TGF- $\beta$   
by both epithelial and connective cells (Tsuji et al.,  
1990). The structural homology data therefore predict  
that the murine LTBP-3 gene shown in FIG. 15B should be  
10 expressed by both epithelial and connective tissue cells.  
Tissue *in situ* hybridization was used to test this  
hypothesis.

An overview of the expression pattern as determined  
15 by tissue *in situ* hybridization is presented in FIG. 17A,  
FIG. 17B, FIG. 17C, and FIG. 17D. Approximate mid-  
sagittal sections of normal murine embryos at days 8.5-  
9.0, 13.5 and 16.5 p.c. of development were hybridized  
with a <sup>35</sup>S-labeled single stranded normal sense riboprobe  
20 from the same cDNA construct was used. At day 8.5-9.0 of  
development, intense gene expression was observed in the  
mesometrial and anti-mesometrial uterine tissues,  
ectoplacental cone, placenta, placental membranes. The  
transcript appeared to be widely expressed in murine  
25 embryo mesenchymal/connective tissue compartments,  
including the facial mesenchyme, at days 8.5-9.0, 13.5  
and 16.5 of development. Particularly intense expression  
of the transcript was noted in the liver.

30 Microscopy of day 8.5-9.0 embryos confirmed the  
widespread expression of the murine gene by mesenchymal  
cells. Significant expression of the transcript by cells  
of the developing central nervous system, somites and

- 135 -

cardiovascular tissue (myocardium plus endocardium) was also observed.

Microscopy of day 13.5 and day 16.5 embryos demonstrated expression of the murine gene by skeletal muscle cells and by cells involved in intramembranous and endochondral bone formation. The transcript was expressed by osteoblasts and by periosteal cells of the calvarium, mandible and maxilla. The transcript was also identified in both cartilage and bone of the lower extremity. A positive signal was detected in perichondrial cells and chondrocytes (proliferating > mature > hypertrophic) of articular cartilage, the presumptive growth plate, and the cartilage model within the central canal. The positive signal was also expressed by blood vessel endothelial cells within the mid-diaphysis, and the surrounding muscle cells (FIG. 18A, FIG. 18B, FIG. 18C, FIG. 18D, FIG. 18E, FIG. 18F, FIG. 18G, FIG. 18H, FIG. 18I, FIG. 18J, FIG. 18K, FIG. 18L, FIG. 18M, FIG. 18N, FIG. 18O, and FIG. 18P).

Respiratory epithelial cells lining developing small airways and connective tissue cells in the pulmonary interstitium expressed the murine transcript, as did myocardial cells (atria and ventricles) and endocardial cushion tissue. Cells within the walls of large arteries also expressed the transcript. Expression of the murine gene was identified in several organs of the alimentary system, including the tongue, esophagus, stomach, small and large intestine, pancreas and liver. Mucosal epithelial cells lining the upper and lower digestive tract plus the smooth muscle and connective tissue cells found in the submucosa expressed the transcript, as did acinar cells of the exocrine pancreas. Despite the high level of transcript expression in the liver, these results suggest both cell populations express the LTBP-3 transcript.

- 136 -

In the kidney, expression above the basal level was observed in cells of developing nephrons, the ureteric bud, kidney blastema and the kidney interstitium. In the skin, epidermal and adnexal keratinocytes, dermal  
5 connective tissue cells, and brown fat cells within the dorsal subcutis expressed the murine transcript. In the central and peripheral nervous systems, ganglion cells within the cerebrum, brainstem, spinal cord, and peripheral nerves expressed the murine transcript. The  
10 transcript was also intensely expressed by cells of the developing murine retina.

Thus, the murine gene is widely expressed by both epithelial and connective tissue cells, a pattern that  
15 would be expected for a latent TGF- $\beta$  binding protein. Three final observations argue that the LTBP-like (LTBP-3) sequence presented in FIG. 25 is not simply the murine homologue of human LTBP. First, domain #4 of the murine LTBP-like (LTBP-3) sequence has a smaller number of EGF-  
20 like repeat motifs than human and rat LTBP (8 versus 11). Second, portions of the human and rat LTBP-like coding sequence were characterized and found to share ~90% identity with human and rat LTBP but only 65% identity with the murine LTBP-like gene. Third, the human LTBP  
25 and LTBP-like genes are localized to separate chromosomes. Human LTBP was assigned to human chromosome 2 based on the analysis of human x rodent somatic cell hybrid lines (Stenman et al., 1994). The present invention represents the first mapping of an LTBP gene in  
30 the murine. The human LTBP-like genes was recently localized to chromosome 11 band q12, while the murine gene was mapped to murine chromosome 19, band B (a region of conserved synten), using several independent approaches, including fluorescent *in situ* hybridization.

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The first indication of alternative splicing came from molecular cloning studies in the murine, in which

- 137 -

independent cDNA clones were isolated with a deletion of 51 bp from the coding sequence. PCR™/Southern blot analysis provided additional evidence that the homologous 51 bp sequence was alternatively spliced in normal murine embryo tissues.

Northern blot analysis also demonstrated that the novel fibrillin gene was also expressed in rat callus three weeks after osteotomy, after mineralization has begun. Gene expression in normal adult rat bone tissue was insignificant, which suggests that microfibrils are an important part of the bone fracture healing response. The novel fibrillin-like gene was expressed in callus as a pair of alternatively spliced transcripts. This result has been independently reproduced on three occasions. Molecular cloning of the novel fibrillin gene in both murine and rat has identified potential splice junction sites for the alternative splicing event.

MC3T3-E1 murine pre-osteoblasts were used to demonstrate that the murine gene product was capable of binding TGF- $\beta$ . MC3T3-E1 cells were utilized because they synthesize and secrete TGF- $\beta$ , which may act as an autocrine regulator of osteoblast proliferation (Amarnani et al., 1993; Van Vlasselaer et al., 1994; Lopez-Casillas et al., 1994).

To determine whether or not MC3T3-E1 cells co-expressed the murine gene product of TGF- $\beta$ , cells were plated on 100-mm dishes under differentiating conditions (Quarles et al., 1992) and the medium was replaced twice weekly. Parallel dishes were plated and assayed for cell number and alkaline phosphatase activity, which confirmed that osteoblast differentiation was indeed taking place. Equal aliquots of total cellular RNA was prepared from these MC3T3-E1 cells after 5, 14 and 28 days in culture for Northern blot analysis. As shown in FIG. 19,

- 138 -

expression of the new murine gene peaked on day 14 of culture. Since MC3T3-E1 cells also show a peak in alkaline phosphatase activity on day 14 of culture (Quarles et al., 1992), the results suggest for the first time that LTBP-2 gene expression is an early marker of osteoblast differentiation.

### C. DISCUSSION

This study reports the molecular cloning of a novel LTBP-like gene that contains numerous EGF-like repeats. Northern analysis indicates that the gene encodes a single transcript of ~4.6 kb in murine embryo tissues. The deduced amino acid sequence of the murine gene product appears to be a secreted polypeptide of 1,251 amino acids. Although it is similar to fibrillin, the overall structural organization and expression pattern of this gene product most resembles LTBP, a latent TGF- $\beta$  binding protein that was originally isolated and characterized by Heldin and co-workers (Kanzaki et al., 1990). Several observations strongly suggest that LTBP and the murine LTBP-like gene product are therefore derived from related but distinct genetic loci. First, LTBP and the LTBP-like coding sequence share ~40% identity and differences exist in the number of EGF-CB repeats in the deduced polypeptide sequence of the two molecules. Second, a portion of the murine LTBP gene has been cloned and shown to share ~90% identity with human and rat LTBP. Conversely, portions of the human and rat LTBP-like genes have been cloned and shown to share ~90% identity with the murine LTBP-like gene. Third, LTBP and the LTBP-like gene reside on different human chromosomes (Stenman et al., 1994). Taken together, these data suggest that a family of at least two LTBP genes exists.

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Similarities in the structural organization of LTBP-1 and the fibrillin-1 and fibrillin-2 polypeptides have



- 139 -

been noted previously (Pereira et al., 1993; Zhang et al., 1994; Taipale et al., 1994). For example, LTBP-1 and the fibrillins are all secreted extracellular matrix constituents. Moreover, each polypeptide can be  
5 organized into five domains, two of which consists predominantly of EGF-CB and TGF- $\beta$  repeat motifs. LTBP-1 and fibrillin-1 also share a domain that is proline-rich, and LTBP possesses an 8-cysteine repeat previously referred to as the "Fib motif" because it was assumed to  
10 be unique to fibrillin (Pereira et al., 1993). These similarities likely explain the initial isolation and cloning of the LTBP-2 PCR™ product, especially since the human oligonucleotide primers used to initially amplify murine cDNA were designed to direct the synthesis of an  
15 EGF-CB repeat in domain #4.

Another point of distinction between LTBP-2 and fibrillin concerns the spacing of conserved cysteines C<sub>4</sub> and C<sub>5</sub> in EGF-like repeats. Fibrillin-1 and fibrillin-2  
20 each contain >50 such repeats, and in every one the spacing is C<sub>4</sub>-X-C<sub>5</sub>. While this pattern is repeated in a majority of the EGF-like repeats in LTBP-1 and LTBP-2, both genes also contain repeats with the spacing C<sub>4</sub>-X-X-C<sub>5</sub>. Although the significance of this observation is  
25 unclear, variation in the number of amino acids between C<sub>4</sub> and C<sub>5</sub> would not be expected to alter the function of the EGF-like repeat. Mature EGF is a 48 amino acid secreted polypeptide consisting of two subdomains that have few interdomain contacts (Engel, 1989; Davis, 1990). The  
30 larger NH<sub>2</sub>-terminal subdomain consists of residues 1-32 and is stabilized by a pair of disulfide bonds (C<sub>1</sub>-C<sub>3</sub> and C<sub>2</sub>-C<sub>4</sub>), whereas the smaller COOH-terminal subdomain (amino acids 33-48) is stabilized by a single disulfide bond (C<sub>5</sub>-C<sub>6</sub>). The COOH-terminal subdomain has a highly conserved  
35 conformation that only is possible if certain residues and the distances between them are well conserved, while conformation-sequence requirements for the NH<sub>2</sub>-terminal

- 140 -

subdomain are relatively relaxed. Variation in C<sub>4</sub>-C<sub>5</sub> spacing would not be expected to alter conformation because these residues do not normally form a disulfide bond and the spacing variation occurs at the interface of subdomains that would not be predicted to interact. The cloning of additional genes will decide whether variation in C<sub>4</sub>-C<sub>5</sub> spacing is a reliable discriminator between members of the LTBP and fibrillin gene families.

10       The LTBP-2 gene is expressed more widely during development than fibrillin-1 or fibrillin-2. Studies in developing murine tissues have shown that the *Fbn-1* gene is expressed by mesenchymal cells of developing connective tissue, whereas the murine LTBP-like gene is  
15       intensely expressed by epithelial, parenchymal and stromal cells. Earlier reports have suggested that TGF- $\beta$  plays a role in differentiation and morphogenesis during murine development (Lyons and Moses, 1990), when TGF- $\beta$  is produced by epithelial, parenchymal and stromal cells.  
20       Tsuji et al., (1990) and others have suggested that the expression of TGF- $\beta$  binding proteins should mirror that of TGF- $\beta$  itself; the expression pattern of the LTBP-2 gene over the course of murine development is consistent with this expectation. However, the LTBP-2 gene may not  
25       be completely co-regulated with TGF- $\beta$ . TGF- $\beta$  gene and protein expression during murine development has been surveyed extensively (Heine et al., 1987; Lehnert and Akhurst, 1988; Pelton et al., 1989; Pelton et al., 1990a,b; Millan et al., 1991); these studies have not  
30       identified expression by skeletal muscle cells, chondrocytes, hepatocytes, ganglion cells, mucosal cells lining the gut, and epithelial cells of developing nephrons. It is conceivable that the LTBP-2 molecule has

- 141 -

an additional function in certain connective tissues besides targeting TGF- $\beta$ .

The binding properties of the LTBP-2 gene product are under investigation. Formally, the LTBP-2 polypeptide may bind a specific TGF- $\beta$  isoform, another member of the TGF- $\beta$  superfamily (e.g., a bone morphogenetic protein, inhibin, activin, or Mullerian inhibiting factor), or a growth factor unrelated to TGF- $\beta$ . Anti-peptide antibodies to the murine LTBP-2 polypeptide have been generated and osteoblast cell lines that express the molecule at relatively high levels have been identified. Studies with these reagents suggest that LTBP-2 assembles intracellularly into large latent complexes with a growth factor that is being characterized by immunological methods.

The presence of dibasic amino acids in the LTBP-2 sequence suggests that it may undergo cell- and tissue-specific proteolysis. TGF- $\beta$  regulates extracellular matrix production by suppressing matrix degradation (through a decrease in the expression of proteases such as collagenase, plasminogen activator, and stromelysin plus an increase in the expression of proteinase inhibitors such as plasminogen activator inhibitor-1 and tissue inhibitor of metalloproteinase-1) and by stimulating matrix macromolecule synthesis (for recent review, see Lyons and Moses, 1990; Massague, 1990; Laiho and Keski-Oja, 1992; Miyazono et al., 1992). Conversely, production of extracellular matrix has been shown to down regulate TGF- $\beta$  gene expression (Streuli et al., 1993). TGF- $\beta$  may therefore regulate extracellular matrix production through a sophisticated feedback loop that influences the expression of a relatively large number of genes. LTBP-1 and LTBP-2 may contribute to this regulation by facilitating the assembly and secretion of large latent growth factor complexes and then targeting

- 142 -

the complex to specific connective tissues (Taipale et al., 1994).

If LTBP-3 is like LTBP-1, it has the potential to function as a secreted, extracellular structural protein. As demonstrated here, domain #1 of LTBP-3 appears to be a unique sequence that likely has a globular conformation. Domain #1 also is highly basic and may facilitate LTBP-2 binding to acidic molecules (e.g., acidic proteoglycans) within the extracellular space. Sequences rich in basic amino acids have also been shown to function as endoproteolytic processing signals for several peptide hormones (Barr, 1991; Steiner et al., 1992). It is possible, therefore, that the NH<sub>2</sub>-terminus of LTBP-3 is proteolytically processed in a tissue-specific manner. Domains #2 and #4 consist of consecutive cysteine-rich repeats, the majority of which are of the EGF-CB type. Besides binding calcium (Corson et al., 1993), these repeats may provide LTBP-3 with regions conformation capable of interacting with other matrix macromolecules (Engel, 1989). Domain #3 is proline rich and may be capable of bending (or functioning like a hinge) in three-dimensional space (MacArthur and Thornton, 1991). (In this regard, domain #2 is of interest because it has a similar stretch of 135 amino acids that is both proline- and glycine-rich. Since glycine-rich sequences are also thought to be capable of bending or functioning like a hinge in three-dimensional space, this amino acid sequence may interrupt the extended conformation of domain #2, thereby providing it with a certain degree of flexibility in three-dimensional space.) Domain #5 also appears to be a unique sequence having a globular conformation. The absence of a known cell attachment motif may indicate that, in contrast to LTBP-1, the LTBP-3 molecule may have a more limited role in the extracellular matrix (i.e., that of a structural protein)

- 143 -

in addition to its ability to target latent TGF- $\beta$  complexes to specific connective tissues.

MC3T3-E1 pre-osteoblasts co-express LTBP-3 and  
5 TGF- $\beta$ 1 and these proteins form a complex in the culture  
medium. These results are particularly interesting  
because bone represents one of the largest known  
repositories of latent TGF- $\beta$  (200  $\mu$ g/kg bone; Seyedin  
et al., 1986 and 1987), and because this growth factor  
10 plays a critical role in the determination of bone  
structure and function. For example, TGF- $\beta$  is thought to  
(i) provide a powerful stimulus to bone formation in  
developing tissues, (ii) function as a possible "coupling  
factor" during bone remodeling (a process that  
15 coordinates bone resorption and formation), and (iii)  
exert a powerful bone inductive stimulus following  
fracture. Activation of the latent complex may be an  
important step governing TGF- $\beta$  effects, and LTBP may  
modulate the activation process (e.g., it may "protect"  
20 small latent complexes from proteolytic attack).

Expression of large latent TGF- $\beta$  complexes bearing  
LTBP may be physiologically relevant to, i.e., may be  
part of the mechanism of, the pre-osteoblast  $\rightarrow$  osteoblast  
25 differentiation cascade. This is based on the evidence  
that MC3T3-E1 cells express large latent TGF- $\beta$ 1 complexes  
bearing LTBP-2 precisely at the time of transition from  
the pre-osteoblast to osteoblast phenotype (~day 14 in  
culture, or, at the onset of alkaline phosphatase  
30 expression; see Quarles et al., 1992). The organ culture  
model, for example, likely is comprised of differentiated  
osteoblasts but few bone progenitors, making it a  
difficult model at best in which to study the  
differentiation cascade (Dallas et al., 1984). It is  
35 also well known that MG63, ROS17/2.8 and UMR 106 cells  
are rapidly dividing and they express the osteoblast  
phenotype. Thus, these osteoblast-like cell lines do not

show the uncoupling of cell proliferation and cell differentiation that characterizes the normal (physiologically relevant) pre-osteoblast → osteoblast transition (Gerstenfeld et al., 1984; Stein and Lian, 1993). Therefore, the production of small versus large latent TGF- $\beta$  complexes may be associated with specific stages in the maturation of bone cells.

LTBP-3 may bind calcium, since EGF-CB repeats have been shown to mediate high affinity calcium binding in LTBP-1 and other proteins (Colosetti et al., 1993). Calcium binding, in turn, may contribute to molecular conformation and the regulation of its interactions with other molecules. The presence of dibasic amino acids suggests that LTBP-3 may also undergo cell- and tissue-specific proteolysis. TGF- $\beta$  regulates extracellular matrix production by suppressing matrix degradation (through a decrease in the expression of proteases such as collagenase, plasminogen activator, and stromelysin plus an increase in the expression of proteinase inhibitors such as plasminogen activator inhibitor-1 and tissue inhibitor of metalloproteinase-1) and by stimulating matrix macromolecule synthesis (for recent reviews, see Lyons and Moses, 1990; Massague, 1990; Laiho and Keski-Oja, 1992; and Miyazono et al., 1993). Conversely, production of extracellular matrix has been shown to down regulate TGF- $\beta$  gene expression (Streuli et al., 1993). TGF- $\beta$  may therefore regulate extracellular matrix production through a sophisticated feedback loop that influences the expression of a relatively large number of genes. LTBP-1, LTBP-2, and LTBP-3 may contribute to this regulation by facilitating the assembly and secretion of large latent growth factor

- 145 -

complexes and then targeting the complex to specific connective tissues (Taipale et al., 1994).

#### EXAMPLE XVI

##### 5     PREPARATION OF ANTIBODIES AGAINST THE LTBP-3 GENE PRODUCT

10     An affinity-purified antibody (#274) capable of immunoprecipitating was prepared against the murine LTBP-3 gene product. A full-length murine cDNA was assembled into the pcDNA3 mammalian expression vector (Invitrogen) and expressed following transient transfection of 293T cells. Nascent polypeptides, radiolabeled by addition of <sup>35</sup>S Cys to the medium of transfected cells, were immunoprecipitated using affinity-purified antibody #274. 15     As shown in FIG. 20, the new murine polypeptide was estimated to be 180-190 kDa. To ensure the specificity of #274 binding, we showed that preincubation with 10 µg of synthetic peptide blocks immunoprecipitation of the 180-190 kDa band.

20     Finally, MC3T3-E1 cells were cultured for 7 days under differentiating conditions and double-labeled with 30 µCi/ml <sup>35</sup>S cysteine and <sup>35</sup>S methionine in deficient media. Radiolabeled media was dialyzed into cold PBS with protease inhibitors. Aliquots of the dialyzed 25     medium sample (10<sup>6</sup> incorporated CPM) were analyzed by a combined immunoprecipitation/Western analysis protocol. The murine polypeptide was clearly and reproducibly secreted by MC3T3-E1 cells, migrating under reducing 30     conditions as a single band of 180-190 kDa (FIG. 21). Consistent with the results of previous studies (e.g., Miyazono et al., 1988; Dallas et al., 1994; Moren et al., 1994), bands of 70 and 50 kDa corresponding to the TGF-β1 precursor were co-immunoprecipitated with the 180 kDa 35     LTBP-3 protein. Weak bands of 40 and 12 kDa were also identified in experiments in which only immunoprecipitation was performed. The latter were not

- 146 -

included in FIG. 21 because they migrated within that portion of the gel included in the Western analysis. Protein bands of 70-12.5 kDa are not variant forms of LTBP-3; FIG. 20 demonstrates that LTBP-3 migrates as a single band of 180-190 kDa following transient transfection of 293T cells, which fail to make TGF- $\beta$ 1. By immunoprecipitation, a unique band consistent with monomeric mature TGF- $\beta$ 1 was found in the LTBP-2 immunoprecipitate. Antibody #274 is incapable of binding TGF- $\beta$ 1 as determined by radioimmunoassay using commercially available reagents (R&D Systems) and the manufacturer's suggested protocols. These results have been reproduced in 6 independent experiments which utilized 3 separate lots of MC3T3-E1 medium. Thus the new murine LTBP-3 polypeptide binds TGF- $\beta$  *in vitro*.

#### EXAMPLE XVII

##### ISOLATION OF A GENE ENCODING MURINE LTBP-2

In addition to determining the DNA and corresponding polypeptide sequence of the murine LTBP-3 gene, the murine LTBP-2 gene was also cloned and sequenced.

The complete cDNA nucleotide sequence for murine LTBP-2 is shown in FIG. 27 (SEQ ID NO:17). The deduced amino acid sequence is shown in FIG. 28 (SEQ ID NO:18).

#### EXAMPLE XVIII

##### 30 EXPRESSION OF RECOMBINANT TYPE II COLLAGEN

The *Pichia* Expression Kit (Invitrogen, Inc.) may be used to prepare recombinant type II collagen. This kit, based on the methylotrophic yeast, *Pichia pastoris*, allows high-level expression of recombinant protein in an easy-to-use relatively inexpensive system. In the absence of the preferred carbon source, glucose, *P.*



- 147 -

*pastoris* utilizes methanol as a carbon source. The AOX1 promoter controls the gene that codes for the expression of the enzyme alcohol oxidase, which catalyzes the first step in the metabolism of methanol. This promoter, which is induced by methanol, has been characterized and incorporated into a series of *Pichia* expression vectors. This feature of *Pichia* has been exploited to express high levels of recombinant proteins often in the range of grams per liter. Because it is eukaryotic, *P. pastoris* utilizes posttranslational modification pathways that are similar to those used by mammalian cells. This implies that the recombinant type II collagen will be glycosylated and will contain disulfide bonds.

The inventors contemplate the following particular elements to be useful in the expression of recombinant type II collagen: the DNA sequence of human type II collagen (SEQ ID NO:11) (Lee et al., 1989); rat type II collagen (SEQ ID NO:13) (Michaelson, et al., 1994); and/or mouse type II collagen (SEQ ID NO:15) (Ortman, et al., 1994). As other sources of DNA sequences encoding type II collagen are available, these three are examples of many sequence elements that may be useful in the present invention.

For preparation of a recombinant type II collagen, the native type II collagen cDNA is modified by the addition of a commercially available epitope tag (the HA epitope, Pharmacia, LKB Biotechnology, Inc.). Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR technology of U.S. Patent 4,603,102 (herein incorporated by reference) or by introducing selected sequences into recombinant vectors for recombinant production. (PCR™ is a registered trademark of Hoffmann-LaRoche, Inc.). This is followed by cloning into the

- 148 -

*Pichia* expression vector. The resulting plasmid is characterized by DNA sequence analysis, linearized by digestion with NotI, and spheroplasts will be prepared and transformed with the linearized construct according to the manufacturer's recommendations.

Transformation facilitates a recombination event in vivo between the 5' and 3' AOX1 sequences in the *Pichia* vector and those in the *Pichia* genome. The result is the replacement of AOX1 with the gene of interest.

Transformants are then plated on histidine-deficient media, which will select for successfully transformed cells. Transformants are further selected against slow growth on growth media containing methanol. Positive transformants are grown for 2 days in liquid culture and then for 2-6 days in broth that uses methanol as the sole carbon source. Protein expression is evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western hybridization using a commercially available polyclonal antisera to the HA epitope (Pharmacia).

Recombinant type II collagen protein can be purified according to the manufacturer's recommendations, dialyzed against double distilled, deionized water and lyophilized in 10 mg aliquots. The aliquots are sterilized and used as implant material for the osteoconductive matrices.

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\* \* \*

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations

- 149 -

may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be

5    apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and

10   modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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- 162 -

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GOLDSTEIN, Steven A.

LIN, Wushan

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FOR STIMULATING BONE CELLS

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(B) COMPUTER: IBM PC compatible

35 (C) OPERATING SYSTEM: PC-DOS/MS-DOS/ASCII

(D) SOFTWARE: PatentIn Release #1.0, Version

#1.30



- 163 -

## (vii) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: UNKNOWN
- (B) FILING DATE: CONCURRENTLY HEREWITH
- (C) CLASSIFICATION: UNKNOWN

5

## (viii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/316,650
- (B) FILING DATE: 30-SEP-1994
- (C) CLASSIFICATION: UNKNOWN

10

- (A) APPLICATION NUMBER: US 08/199,780
- (B) FILING DATE: 18-FEB-1994
- (C) CLASSIFICATION: UNKNOWN

15

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25

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 417 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30

## (ii) MOLECULE TYPE: protein

-164-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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 1 5 10 15

5 Leu Leu Gly Gly Ala Thr Asp Ala Ser Leu Met Pro Glu Thr Gly Lys  
 20 25 30

10 Lys Lys Val Ala Glu Ile Gln Gly His Ala Gly Gly Arg Arg Ser Gly  
 35 40 45

Gln Ser His Glu Leu Leu Arg Asp Phe Glu Ala Thr Leu Leu Gln Met  
 50 55 60

15 Phe Gly Leu Arg Arg Arg Pro Gln Pro Ser Lys Ser Ala Val Ile Pro  
 65 70 75 80

Asp Tyr Met Ser Asp Leu Tyr Arg Leu Gln Ser Gly Glu Glu Glu  
 85 90 95

20 Glu Glu Gln Ser Gln Gly Thr Gly Leu Glu Tyr Pro Glu Arg Pro Ala  
 100 105 110

-165-

Ser Ser Ala Asn Thr Val Ser Ser Phe His His Glu Glu His Leu Glu  
 115 120 125  
 Asn Ile Pro Gly Thr Ser Glu Ser Ser Ala Phe Arg Phe Phe Asn  
 130 135 140  
 Leu Ser Ser Ile Pro Glu Asn Glu Val Ile Ser Ser Ala Glu Leu Arg  
 145 150 155 160  
 Leu Phe Arg Glu Gln Val Asp Gln Gly Pro Asp Trp Glu Gln Gly Phe  
 165 170 175  
 His Arg Met Asn Ile Tyr Glu Val Met Lys Pro Pro Ala Glu Met Val  
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 Pro Gly His Leu Ile Thr Arg Leu Leu Asp Thr Ser Leu Val Arg His  
 195 200 205  
 Asn Val Thr Arg Trp Glu Thr Phe Asp Val Ser Pro Ala Val Leu Arg  
 210 215 220  
 Trp Thr Arg Glu Lys Gln Pro Asn Tyr Gly Leu Ala Ile Glu Val Thr  
 225 230 235 240

-166-

His Leu His Gln Thr Arg Thr His Gln Gly Gln His Val Ser Ile Ser  
 245 250 255  
 Arg Ser Leu Pro Gln Gly Ser Gly Asn Trp Ala Gln Leu Arg Pro Leu  
 260 265 270  
 Leu Val Thr Phe Gly His Asp Gly Arg Gly His Thr Leu Thr Arg Arg  
 275 280 285  
 Ser Ala Lys Arg Ser Pro Lys His His Pro Gln Arg Ser Ser Lys Lys  
 290 295 300  
 Asn Lys Asn Cys Arg Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val  
 305 310 315 320  
 Gly Trp Asn Asp Trp Ile Val Ala Pro Pro Gly Tyr Gln Ala Phe Tyr  
 325 330 335  
 Cys His Gly Asp Cys Pro Phe Pro Leu Ala Asp His Leu Asn Ser Thr  
 340 345 350  
 Asn His Ala Ile Val Gln Thr Leu Val Asn Ser Val Asn Ser Ser Ile  
 355 360 365

Pro Lys Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile Ser Met Leu  
 370 375 380  
 Tyr Leu Asp Glu Tyr Asp Lys Val Val Leu Lys Asn Tyr Gln Glu Met  
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10 Ala

(2) INFORMATION FOR SEQ ID NO:2:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3753 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..3753

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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	20	25	30		
	CTG GGC CCC GGC GGC CGA GGG GTG GGC CGG CCG GGC AGC GGC GCA CAG				96
	Leu Gly Pro Gly Gly Arg Gly Val Gly Arg Pro Gly Ser Gly Ala Gln				
	35	40	45		
15	GCG GGG GCG GGG CGC TGG GCC CAA CGC TTC AAG GTG GTC TTT GCG CCT				144
	Ala Gly Ala Gly Arg Trp Ala Gln Arg Phe Lys Val Val Phe Ala Pro				
	50	55	60		
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-169-

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480 20 ACA GGC CCG CTG CCG CCC CTT GCC CCA GAA GGA GAG TCT GTG GCT AGC  
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-170-

528

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624

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672

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720

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768

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-171-

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 Leu Pro Gly Leu Thr Lys Gln Glu Asp Cys Cys Gly Ser Ile Gly Thr 275 280 285  
  
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-172-

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1296  
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-174-

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 565 570 575

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-175-

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-176-

2256

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5 ATA CGA ACC CGC ACA GGA CGC CTC AGT TGC ATA GAC GTG GAT GAC TGT  
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 805 810 815

20 GGG GGT GAC TGC ATC AAT ACC AAT GGT TCC TAC AGA TGT CTC TGT CCC  
 Gly Gly Asp Cys Ile Asn Thr Asn Gly Ser Tyr Arg Cys Leu Cys Pro  
 820 825 830

2496

-177-

2544

CTG GGT CAT CGG TTG GTG GGC GGC AAG AAG TGC AAG AAA GAT ATA GAT  
 Leu Gly His Arg Leu Val Gly Gly Arg Lys Cys Lys Lys Asp Ile Asp  
 835 840 845

2592

5 GAG TGC AGC CAG GAC CCA GGC CTG TGC CTG CCC CAT GCC TGC GAG AAC  
 Glu Cys Ser Gln Asp Pro Gly Leu Cys Leu Pro His Ala Cys Glu Asn  
 850 855 860

2640

10 CTC CAG GGC TCC TAT GTC TGT GTC TGT GAT GAG GGT TTC ACA CTC ACC  
 Leu Gln Gly Ser Tyr Val Cys Val Cys Asp Glu Gly Phe Thr Leu Thr  
 865 870 875 880

2688

15 CAG GAC CAG CAT GGG TGT GAG GAG GTG GAG CAG CCC CAC CAC AAG AAG  
 Gln Asp Gln His Gly Cys Glu Glu Val Glu Gln Pro His His Lys Lys  
 885 890 895

2736

GAG TGC TAC CTT AAC TTC GAT GAC ACA GTG TTC TGT GAC AGC GTA TTG  
 Glu Cys Tyr Leu Asn Phe Asp Asp Thr Val Phe Cys Asp Ser Val Leu  
 900 905 910

2784

20 GCT ACC AAT GTC ACT CAG CAG GAA TGC TGT TGC TCT CTG GGA GCT GGC  
 Ala Thr Asn Val Thr Gln Gln Glu Cys Cys Ser Leu Gly Ala Gly  
 915 920 925

-178-

2832  
TGG GGA GAC CAC TGC GAA ATC TAT CCC TGT CCA GTC TAC AGC TCA GCC  
Trp Gly Asp His Cys Glu Ile Tyr Pro Cys Pro Val Tyr Ser Ser Ala  
930 935 940

5 GAA TTT CAC AGC CTG GTG CCT GAT GGG AAA AGG CTA CAC TCA GGA CAA 2880  
Glu Phe His Ser Leu Val Pro Asp Gly Lys Arg Leu His Ser Gly Gln  
945 950 955 960

10 CAA CAT TGT GAA CTA TGC ATT CCT GCC CAC CGT GAC ATC GAC GAA TGC 2928  
Gln His Cys Glu Leu Cys Ile Pro Ala His Arg Asp Ile Asp Glu Cys  
965 970 975

15 ATA TTG TTT GGG GCA GAG ATC TGC AAG GAG GGC AAG TGT GTG AAC TCG 2976  
Ile Leu Phe Gly Ala Glu Ile Cys Lys Glu Gly Lys Cys Val Asn Ser  
980 985 990

3024  
CAG CCC GGC TAC GAG TGC TAC TGC AAG CAG GGC TTC TAC TAC GAT GGC  
Gln Pro Gly Tyr Glu Cys Tyr Cys Lys Gln Gly Phe Tyr Tyr Asp Gly  
995 1000 1005

20 AAC CTG CTG GAG TGC GTG GAC GTG GAC GAG TGC TTG GAT GAG TCT AAC 3072  
Asn Leu Leu Glu Cys Val Asp Val Asp Glu Cys Leu Asp Glu Ser Asn  
1010 1015 1020



-179-

3120  
TGC AGG AAC GGA GTG TGT GAG AAC ACG TGG CGG CTA CCG TGT GCC TGC  
Cys Arg Asn Gly Val Cys Glu Asn Thr Trp Arg Leu Pro Cys Ala Cys  
1025 1030 1035 1040

3168  
5 ACT CCG CCG GCA GAG TAC AGT CCC GCA CAG GCC CAG TGT CTG ATC CCG  
Thr Pro Pro Ala Glu Tyr Ser Pro Ala Gln Ala Gln Cys Leu Ile Pro  
1045 1050 1055

3216  
10 GAG AGA TGG AGC ACG CCC CAG AGA GAC GTG AAG TGT GCT GGG GCC AGC  
Glu Arg Trp Ser Thr Pro Gln Arg Asp Val Lys Cys Ala Gly Ala Ser  
1060 1065 1070

3264  
GAG GAG AGG ACG ACG GCA TGT GTA TGG GGC CCC TGG GCG GGA CCT GCC CTC  
Glu Glu Arg Thr Ala Cys Val Trp Gly Pro Trp Ala Gly Pro Ala Leu  
1075 1080 1085

3312  
15 ACT TTT GAT GAC TGC TGC TGC CGC CAG CCG CGG CTG GGT ACC CAG TGC  
Thr Phe Asp Asp Cys Cys Cys Arg Gln Pro Arg Leu Gly Thr Gln Cys  
1090 1095 1100

3360  
20 AGA CCG TGC CCG CCA CGT GGC ACC GGG TCC CAG TGC CCG ACT TCA CAG  
Arg Pro Cys Pro Pro Arg Gly Thr Gly Ser Gln Cys Pro Thr Ser Gln  
1105 1110 1115 1120

-180-

3408

AGT GAG AGC AAT TCT TTC TGG GAC ACA AGC CCC CTG CTA CTG GGG AAG  
 Ser Glu Ser Asn Ser Phe Trp Asp Thr Ser Pro Leu Leu Gly Lys  
 1125 1130 1135

3456

TCT CCG CGA GAC GAA GAC AGC TCA GAG GAG GAT TCA GAT GAG TGC CGT  
 Ser Pro Arg Asp Glu Asp Ser Ser Glu Glu Asp Ser Asp Glu Cys Arg  
 1140 1145 1150

3504

TGT GTG AGC GGA CCG TGT GTG CCA CGG CCA GGC GCG GTA TGC GAG  
 Cys Val Ser Gly Pro Cys Val Pro Arg Pro Gly Ala Val Cys Glu  
 1155 1160 1165

3552

TGT CCT GGA GGC TTT CAG CTG GAC GCC TCC CGT GCC CGC TGC GTG GAC  
 Cys Pro Gly Gly Phe Gln Leu Asp Ala Ser Arg Ala Arg Cys Val Asp  
 1170 1175 1180

3600

ATT GAT GAG TGC CGA GAA CTG AAC CAG CGG GGA CTG CTG TGT AAG AGC  
 Ile Asp Glu Cys Arg Glu Leu Asn Gln Arg Gly Leu Cys Lys Ser  
 1185 1190 1195 1200

3648

GAG CGG TGC GTG AAC ACC AGT GGA TCC TTC CGC TGT GTC TGC AAA GCT  
 Glu Arg Cys Val Asn Thr Ser Gly Ser Phe Arg Cys Val Cys Lys Ala  
 1205 1210 1215

20

-181-

3696

GGC TTC ACG CGC AGC CGC CCT CAC GGC CCT GCG TGC CTC AGC GCC GCC  
Gly Phe Thr Arg Ser Arg Pro His Gly Pro Ala Cys Leu Ser Ala Ala  
1220 1225 1230

3744

5 GCT GAT GAT GCA GCC ATA GCC CAC ACC TCA GTG ATC GAT CAT CGA GGG  
Ala Asp Asp Ala Ala Ile Ala His Thr Ser Val Ile Asp His Arg Gly  
1235 1240 1245

3753

TAT TTT CAC

10 Tyr Phe His

1250

## (2) INFORMATION FOR SEQ ID NO:3:

15

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1251 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Arg Gln Ala Ala Leu Gly Leu Leu Ala Leu Leu Leu Ala Leu  
 1 5 10 15  
 Leu Gly Pro Gly Gly Arg Gly Val Gly Arg Pro Gly Ser Gly Ala Gln  
 5 20 25 30  
 Ala Gly Ala Gly Arg Trp Ala Gln Arg Phe Lys Val Val Phe Ala Pro  
 35 40 45  
 Val Ile Cys Lys Arg Thr Cys Leu Lys Gly Gln Cys Arg Asp Ser Cys  
 50 55 60  
 Gln Gln Gly Ser Asn Met Thr Leu Ile Gly Glu Asn Gly His Ser Thr  
 65 70 75 80  
 Asp Thr Leu Thr Gly Ser Ala Phe Arg Val Val Val Cys Pro Leu Pro  
 85 90 95  
 Cys Met Asn Gly Gly Gln Cys Ser Ser Arg Asn Gln Cys Leu Cys Pro  
 100 105 110  
 Pro Asp Phe Thr Gly Arg Phe Cys Gln Val Pro Ala Ala Gly Thr Gly  
 115 120 125

-183-

Ala Gly Thr Gly Ser Ser Gly Pro Gly Trp Pro Asp Arg Ala Met Ser  
 130 135 140  
 Thr Gly Pro Leu Pro Pro Leu Ala Pro Glu Gly Glu Ser Val Ala Ser  
 5 145 150 155 160  
 Lys His Ala Ile Tyr Ala Val Gln Val Ile Ala Asp Pro Pro Gly Pro  
 165 170 175  
 Gly Glu Gly Pro Pro Ala Gln His Ala Ala Phe Leu Val Pro Leu Gly  
 180 185 190  
 Pro Gly Gln Ile Ser Ala Glu Val Gln Ala Pro Pro Val Val Asn  
 195 200 205  
 Val Arg Val His His Pro Pro Gly Ala Ser Val Gln Val His Arg Ile  
 210 215 220  
 Glu Gly Pro Asn Ala Glu Gly Pro Ala Ser Ser Gln His Leu Leu Pro  
 225 230 235 240  
 His Pro Lys Pro Pro His Pro Arg Pro Pro Thr Gln Lys Pro Leu Gly  
 245 250 255

-184-

Arg Cys Phe Gln Asp Thr Leu Pro Lys Gln Pro Cys Gly Ser Asn Pro  
260 265 270

Leu Pro Gly Leu Thr Lys Gln Glu Asp Cys Cys Gly Ser Ile Gly Thr  
275 280 285

Ala Trp Gly Gln Ser Lys Cys His Lys Cys Pro Gln Leu Gln Tyr Thr  
290 295 300

Gly Val Gln Lys Pro Val Pro Val Arg Gly Glu Val Gly Ala Asp Cys  
305 310 315 320

Pro Gln Gly Tyr Lys Arg Leu Asn Ser Thr His Cys Gln Asp Ile Asn  
325 330 335

Glu Cys Ala Met Pro Gly Asn Val Cys His Gly Asp Cys Leu Asn Asn  
340 345 350

Pro Gly Ser Tyr Arg Cys Val Cys Pro Pro Gly His Ser Leu Gly Pro  
355 360 365

Leu Ala Ala Gln Cys Ile Ala Asp Lys Pro Glu Glu Lys Ser Leu Cys  
370 375 380

-185-

Phe Arg Leu Val Ser Thr Glu His Gln Cys Gln His Pro Leu Thr Thr  
 385 390 395 400

Arg Leu Thr Arg Gln Leu Cys Cys Ser Val Gly Lys Ala Trp Gly  
 405 410 415

Ala Arg Cys Gln Arg Cys Pro Ala Asp Gly Thr Ala Ala Phe Lys Glu  
 420 425 430

Ile Cys Pro Gly Trp Glu Arg Val Pro Tyr Pro His Leu Pro Pro Asp  
 435 440 445

Ala His His Pro Gly Gly Lys Arg Leu Leu Pro Leu Pro Ala Pro Asp  
 450 455 460

Gly Pro Pro Lys Pro Gln Gln Leu Pro Glu Ser Pro Ser Arg Ala Pro  
 465 470 475 480

Pro Leu Glu Asp Thr Glu Glu Glu Arg Gly Val Thr Met Asp Pro Pro  
 485 490 495

Val Ser Glu Glu Arg Ser Val Gln Gln Ser His Pro Thr Thr Thr Thr  
 500 505 510

-186-

Ser Pro Pro Arg Pro Tyr Pro Glu Leu Ile Ser Arg Pro Ser Pro Pro  
515 520 525

Thr Phe His Arg Phe Leu Pro Asp Leu Pro Pro Ser Arg Ser Ala Val  
530 535 540

Glu Ile Ala Pro Thr Gln Val Thr Glu Thr Asp Glu Cys Arg Leu Asn  
545 550 555 560

Gln Asn Ile Cys Gly His Gly Gln Cys Val Pro Gly Pro Ser Asp Tyr  
565 570 575

Ser Cys His Cys Asn Ala Gly Tyr Arg Ser His Pro Gln His Arg Tyr  
580 585 590

Cys Val Asp Val Asn Glu Cys Glu Ala Glu Pro Cys Gly Pro Gly Lys  
595 600 605

Gly Ile Cys Met Asn Thr Gly Gly Ser Tyr Asn Cys His Cys Asn Arg  
610 615 620

Gly Tyr Arg Leu His Val Gly Ala Gly Arg Ser Cys Val Asp Leu  
625 630 635 640



-187-

Asn Glu Cys Ala Lys Pro His Leu Cys Gly Asp Gly Gly Phe Cys Ile  
645 650 655

Asn Phe Pro Gly His Tyr Lys Cys Asn Cys Tyr Pro Gly Tyr Arg Leu  
660 665 670

5

Lys Ala Ser Arg Pro Pro Ile Cys Glu Asp Ile Asp Glu Cys Arg Asp  
675 680 685

Pro Ser Thr Cys Pro Asp Gly Lys Cys Glu Asn Lys Pro Gly Ser Phe  
690 695 700

Lys Cys Ile Ala Cys Gln Pro Gly Tyr Arg Ser Gln Gly Gly Ala  
705 710 715 720

15

Cys Arg Asp Val Asn Glu Cys Ser Glu Gly Thr Pro Cys Ser Pro Gly  
725 730 735

Trp Cys Glu Lys Leu Pro Gly Ser Tyr Arg Cys Thr Cys Ala Gln Gly  
740 745 750

20

Ile Arg Thr Arg Thr Gly Arg Leu Ser Cys Ile Asp Val Asp Asp Cys  
755 760 765

-188-

Glu Ala Gly Lys Val Cys Gln Asp Gly Ile Cys Thr Asn Thr Pro Gly  
770 775 780

Ser Phe Gln Cys Gln Cys Leu Ser Gly Tyr His Leu Ser Arg Asp Arg  
5 785 790 795 800

Ser Arg Cys Glu Asp Ile Asp Glu Cys Asp Phe Pro Ala Ala Cys Ile  
805 810 815

Gly Gly Asp Cys Ile Asn Thr Asn Gly Ser Tyr Arg Cys Leu Cys Pro  
10 820 825 830

Leu Gly His Arg Leu Val Gly Gly Arg Lys Cys Lys Lys Asp Ile Asp  
835 840 845

Glu Cys Ser Gln Asp Pro Gly Leu Cys Leu Pro His Ala Cys Glu Asn  
15 850 855 860

Leu Gln Gly Ser Tyr Val Cys Val Cys Asp Glu Gly Phe Thr Leu Thr  
20 865 870 875 880

Gln Asp Gln His Gly Cys Glu Glu Val Glu Gln Pro His His Lys Lys  
885 890 895

-189-

Glu Cys Tyr Leu Asn Phe Asp Asp Thr Val Phe Cys Asp Ser Val Leu  
900 905 910

Ala Thr Asn Val Thr Gln Gln Glu Cys Cys Ser Leu Gly Ala Gly  
915 920 925

5 Trp Gly Asp His Cys Glu Ile Tyr Pro Cys Pro Val Tyr Ser Ser Ala  
930 935 940

10 Glu Phe His Ser Leu Val Pro Asp Gly Lys Arg Leu His Ser Gly Gln  
945 950 955 960

Gln His Cys Glu Leu Cys Ile Pro Ala His Arg Asp Ile Asp Glu Cys  
965 970 975

15 Ile Leu Phe Gly Ala Glu Ile Cys Lys Glu Gly Lys Cys Val Asn Ser  
980 985 990

Gln Pro Gly Tyr Glu Cys Tyr Cys Lys Gln Gly Phe Tyr Tyr Asp Gly  
995 1000 1005

Asn Leu Leu Glu Cys Val Asp Val Asp Glu Cys Leu Asp Glu Ser Asn  
1010 1015 1020

-190-

Cys Arg Asn Gly Val Cys Glu Asn Thr Trp Arg Leu Pro Cys Ala Cys  
1025 1030 1035 1040

Thr Pro Pro Ala Glu Tyr Ser Pro Ala Gln Ala Cys Leu Ile Pro  
1045 1050 1055

5

Glu Arg Trp Ser Thr Pro Gln Arg Asp Val Lys Cys Ala Gly Ala Ser  
1060 1065 1070

Glu Glu Arg Thr Ala Cys Val Trp Gly Pro Trp Ala Gly Pro Ala Leu  
1075 1080 1085

Thr Phe Asp Asp Cys Cys Arg Gln Pro Arg Leu Gly Thr Gln Cys  
1090 1095 1100

15

Arg Pro Pro Pro Arg Gly Thr Gly Ser Gln Cys Pro Thr Ser Gln  
1105 1110 1115 1120

Ser Glu Ser Asn Ser Phe Trp Asp Thr Ser Pro Leu Leu Gly Lys  
1125 1130 1135

20

Ser Pro Arg Asp Glu Asp Ser Ser Glu Glu Asp Ser Asp Glu Cys Arg  
1140 1145 1150

-191-

Cys Val Ser Gly Pro Cys Val Pro Arg Pro Gly Gly Ala Val Cys Glu  
 1155 1160 1165

Cys Pro Gly Gly Phe Gln Leu Asp Ala Ser Arg Ala Arg Cys Val Asp  
 5 1170 1175 1180

Ile Asp Glu Cys Arg Glu Leu Asn Gln Arg Gly Leu Leu Cys Lys Ser  
 1185 1190 1195 1200

Glu Arg Cys Val Asn Thr Ser Gly Ser Phe Arg Cys Val Cys Lys Ala  
 1205 1210 1215

Gly Phe Thr Arg Ser Arg Pro His Gly Pro Ala Cys Leu Ser Ala Ala  
 1220 1225 1230

Ala Asp Asp Ala Ala Ile Ala His Thr Ser Val Ile Asp His Arg Gly  
 1235 1240 1245

Tyr Phe His  
 20 1250

(2) INFORMATION FOR SEQ ID NO:4:

- 192 -

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

5 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

10

AACATGACGC TCATCGGAGA GAAC

24

(2) INFORMATION FOR SEQ ID NO:5:

15

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

25 AGGTGATCGC AGATCCTC

18

(2) INFORMATION FOR SEQ ID NO:6:

30

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: DNA (genomic)

- 193 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TACCGATGCT ACCGCAGCAA TCTT

24

5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

10

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGCCTAAAC TCTACCAGCA CG

22

20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

25

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAGTCACGTC ATCCATTCCA CA

22

35

- 194 -

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

10

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGTCCAAGTT GTGTCTTAGC AG

22

15

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 53 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide



-195-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gly Pro Pro Gly Pro Gln Gly Ala Thr Gly Pro Leu Gly Pro Lys Gly  
 1 5 10 15

5

Gln Thr Gly Glu Pro Gly Ile Ala Gly Phe Lys Gly Glu Gln Gly Pro  
 20 25 30

Lys Gly Glu Thr Gly Pro Ala Gly Pro Gln Gly Ala Pro Gly Pro Ala  
 35 40 45

10

Gly Glu Glu Gly Lys  
 50

15 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 159 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA (genomic)

-196-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGCCCTCCCG GTCCTCAAGG TGCAACTGGT CCTCTGGGCC CCAAAGGTCA GACGGGTGAG 60  
CCCGGCATCG CTGGCTTCAA AGGTGAACAA GGCCCCAAGG GAGAGACTGG ACCTGCTGGG 120  
CCCCAGGGAG CCCCTGGCCC TGCTGGTGAA GAAGGAAAA 159

10 (2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1442 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

20

-197-

Met Ile Arg Leu Gly Ala Pro Gln Ser Leu Val Leu Leu Thr Leu Leu  
1 5 10 15

Ile Ala Ala Val Leu Arg Cys Gln Gly Gln Asp Ala Gln Glu Ala Gly  
20 25 30

Ser Cys Leu Gln Asn Gly Gln Arg Tyr Lys Asp Lys Asp Val Trp Lys  
35 40 45

Pro Ser Ser Cys Arg Ile Cys Val Cys Asp Thr Gly Asn Val Leu Cys  
50 55 60

Asp Asp Ile Ile Cys Glu Asp Pro Asp Cys Leu Asn Pro Glu Ile Pro  
65 70 75 80

Phe Gly Glu Cys Cys Pro Ile Cys Pro Ala Asp Leu Ala Thr Ala Ser  
85 90 95

Gly Arg Lys Leu Gly Pro Lys Gly Gln Lys Gly Glu Pro Gly Asp Ile  
100 105 110

Arg Asp Gly Pro Ala Gly Glu Gln Gly Pro Arg Gly Asp Arg Gly Asp  
115 120 125

-198-

Lys Gly Glu Lys Asn Phe Ala Ala Gln Met Ala Gly Gly Tyr Asp Glu  
 130 135 140  
 5

Lys Ala Gly Gly Ala Gln Met Gly Val Met Gln Gly Pro Met Gly Pro  
 145 150 155 160  
 Met Gly Pro Arg Gly Pro Pro Gly Pro Ala Gly Ala Pro Gly Pro Gln  
 165 170 175  
 10

Gly Phe Gln Gly Asn Pro Gly Glu Pro Gly Glu Pro Gly Val Ser Gly  
 180 185 190  
 Pro Met Gly Pro Arg Gly Pro Pro Gly Pro Ala Gly Lys Pro Gly Asp  
 195 200 205  
 15

Asp Gly Glu Ala Gly Lys Pro Gly Lys Ser Gly Glu Arg Gly Leu Pro  
 210 215 220  
 Gly Pro Met Gly Ala Arg Gly Phe Pro Gly Thr Pro Gly Leu Pro Gly  
 225 230 235 240  
 Val Lys Gly His Arg Gly Tyr Pro Gly Leu Asp Gly Ala Lys Gly Glu  
 245 250 255  
 20

-199-

Ala Gly Ala Pro Gly Val Lys Gly Gly Ser Gly Ser Pro Gly Glu Asn  
260 265 270

Gly Ser Pro Gly Pro Met Gly Pro Arg Gly Leu Pro Gly Glu Arg Gly  
275 280 285

Arg Thr Gly Pro Ala Gly Ala Ala Gly Ala Arg Gly Asn Asp Gly Gln  
290 295 300

Pro Gly Pro Ala Gly Pro Pro Gly Pro Val Gly Pro Ala Gly Gly Pro  
305 310 315 320

Gly Phe Pro Gly Ala Pro Gly Ala Lys Gly Glu Ala Gly Pro Thr Gly  
325 330 335

Ala Arg Gly Pro Glu Gly Ala Gln Gly Ser Arg Gly Glu Pro Gly Asn  
340 345 350

Pro Gly Ser Pro Gly Pro Ala Gly Ala Ser Gly Asn Pro Gly Thr Asp  
355 360 365

Gly Ile Pro Gly Ala Lys Gly Ser Ala Gly Ala Pro Gly Ile Ala Gly  
370 375 380

-200-

Ala Pro Gly Phe Pro Gly Pro Arg Gly Pro Gly Pro Gln Gly Ala  
385 390 395 400

Thr Gly Pro Leu Gly Pro Lys Gly Gln Ala Gly Glu Pro Gly Ile Ala  
405 410 415

5

Gly Phe Lys Gly Asp Gln Gly Pro Lys Gly Glu Thr Gly Pro Ala Gly  
420 425 430

Pro Gln Gly Ala Pro Gly Pro Ala Gly Glu Glu Gly Lys Arg Gly Ala  
435 440 445

10

Arg Gly Glu Pro Gly Gly Ala Gly Pro Ile Gly Pro Pro Gly Glu Arg  
450 455 460

15

Gly Ala Pro Gly Asn Arg Gly Phe Pro Gly Gln Asp Gly Leu Ala Gly  
465 470 475 480

Pro Lys Gly Ala Pro Gly Glu Arg Gly Pro Ser Gly Leu Ala Gly Pro  
485 490 495

20

Lys Gly Ala Asn Gly Asp Pro Gly Arg Pro Gly Glu Pro Gly Leu Pro  
500 505 510

-201-

Gly Ala Arg Gly Leu Thr Gly Arg Pro Gly Asp Ala Gly Pro Gln Gly  
 515 520 525  
 Lys Val Gly Pro Ser Gly Ala Pro Gly Glu Asp Gly Arg Pro Gly Pro  
 530 535 540  
 Pro Gly Pro Gln Gly Ala Arg Gly Gln Pro Gly Val Met Gly Phe Pro  
 545 550 555 560  
 Gly Pro Lys Gly Ala Asn Gly Glu Pro Gly Lys Ala Gly Glu Lys Gly  
 565 570 575  
 Leu Ala Gly Ala Pro Gly Leu Arg Gly Leu Pro Gly Lys Asp Gly Glu  
 580 585 590  
 Thr Gly Ala Ala Gly Pro Pro Gly Pro Ser Gly Pro Ala Gly Glu Arg  
 595 600 605  
 Gly Glu Gln Gly Ala Pro Gly Pro Ser Gly Phe Gln Gly Leu Pro Gly  
 610 615 620  
 Pro Pro Gly Pro Pro Gly Glu Gly Gly Lys Gln Gly Asp Gln Gly Ile  
 625 630 635 640

-202-

Pro Gly Glu Ala Gly Ala Pro Gly Leu Val Gly Pro Arg Gly Glu Arg  
645 650 655

Gly Phe Pro Gly Glu Arg Gly Ser Pro Gly Ala Gln Gly Leu Gln Gly  
660 665 670

Pro Arg Gly Leu Pro Gly Thr Pro Gly Thr Asp Gly Pro Lys Gly Ala  
675 680 685

Ala Gly Pro Asp Gly Pro Pro Gly Ala Gln Gly Pro Pro Gly Leu Gln  
690 695 700

Gly Met Pro Gly Glu Arg Gly Ala Ala Gly Ile Ala Gly Pro Lys Gly  
705 710 715 720

Asp Arg Gly Asp Val Gly Glu Lys Gly Pro Glu Gly Ala Pro Gly Lys  
725 730 735

Asp Gly Gly Arg Gly Leu Thr Gly Pro Ile Gly Pro Pro Gly Pro Ala  
740 745 750

Gly Ala Asn Gly Glu Lys Gly Glu Ala Gly Pro Pro Gly Pro Ser Gly  
755 760 765

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- 203 -

Ser Thr Gly Ala Arg Gly Ala Pro Gly Glu Pro Gly Glu Thr Gly Pro  
 770 775 780  
 Pro Gly Pro Ala Gly Phe Ala Gly Pro Pro Gly Ala Asp Gly Gln Pro  
 785 790 795 800  
 Gly Ala Lys Gly Asp Gln Gly Glu Ala Gly Gln Lys Gly Asp Ala Gly  
 805 810 815  
 Ala Pro Gly Pro Gln Gly Pro Ser Gly Ala Pro Gly Pro Gln Gly Pro  
 820 825 830  
 Thr Gly Val Thr Gly Pro Lys Gly Ala Arg Gly Ala Gln Gly Pro Pro  
 835 840 845  
 Gly Ala Thr Gly Phe Pro Gly Ala Ala Gly Arg Val Gly Pro Pro Gly  
 850 855 860  
 Ala Asn Gly Asn Pro Gly Pro Ala Gly Pro Pro Gly Pro Ala Gly Lys  
 865 870 875 880  
 Asp Gly Pro Lys Gly Val Arg Gly Asp Ser Gly Pro Pro Gly Arg Ala  
 885 890 895

- 204 -

Gly Asp Pro Gly Leu Glu Gly Pro Ala Gly Ala Pro Gly Glu Lys Gly  
 900 905 910

Glu Pro Gly Asp Asp Gly Pro Ser Gly Leu Asp Gly Pro Pro Gly Pro  
 915 920 925

Gln Gly Leu Ala Gly Gln Arg Gly Ile Val Gly Leu Pro Gly Gln Arg  
 930 935 940

Gly Glu Arg Gly Phe Pro Gly Leu Pro Gly Pro Ser Gly Glu Pro Gly  
 945 950 955 960

Lys Gln Gly Ala Pro Gly Ala Ser Gly Asp Arg Gly Pro Pro Gly Pro  
 965 970 975

Val Gly Pro Pro Gly Leu Thr Gly Pro Ala Gly Glu Pro Gly Arg Glu  
 980 985 990

Gly Ser Pro Gly Ala Asp Gly Pro Pro Gly Arg Asp Gly Ala Ala Gly  
 995 1000 1005

Val Lys Gly Asp Arg Gly Glu Thr Gly Ala Leu Gly Ala Pro Gly Ala  
 1010 1015 1020

5

10

15

20

-205-

Pro Gly Pro Pro Gly Ser Pro Gly Pro Ala Gly Pro Thr Gly Lys Gln  
1025 1030 1035 1040

Gly Asp Arg Gly Glu Ala Gly Ala Gln Gly Pro Met Gly Pro Ser Gly  
1045 1050 1055

5

Pro Ala Gly Ala Arg Gly Ile Ala Gly Pro Gln Gly Pro Arg Gly Asp  
1060 1065 1070

Lys Gly Glu Ser Gly Glu Gln Gly Glu Arg Gly Leu Lys Gly His Arg  
1075 1080 1085

10

Gly Phe Thr Gly Leu Gln Gly Leu Pro Gly Pro Gly Pro Ser Gly  
1090 1095 1100

15

Asp Gln Gly Ala Ser Gly Pro Ala Gly Pro Ser Gly Pro Arg Gly Pro  
1105 1110 1115 1120

Pro Gly Pro Val Gly Pro Ser Gly Lys Asp Gly Ser Asn Gly Ile Pro  
1125 1130 1135

20

Gly Pro Ile Gly Pro Pro Gly Pro Arg Gly Arg Ser Gly Glu Thr Gly  
1140 1145 1150

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10

Leu Asn Asn Gln Ile Glu Ser Ile Arg Ser Pro Asp Gly Ser Arg Lys  
1220 1225 1230

Asn Pro Ala Arg Thr Cys Gln Asp Leu Lys Leu Cys His Pro Glu Trp

1235 1240 1245

Lys Ser Gly Asp Tyr Trp Ile Asp Pro Asn Gln Gly Cys Thr Leu Asp  
1250 1255 1260

Ala Met Lys Val Phe Cys Asn Met Glu Thr Gly Glu Thr Cys Val Tyr  
1265 1270 1275 1280

-207-

Pro Asn Pro Ala Thr Val Pro Arg Lys Asn Trp Trp Ser Ser Lys Ser  
1285 1290 1295

Lys Glu Lys Lys His Ile Trp Phe Gly Glu Thr Met Asn Gly Gly Phe  
1300 1305 1310

5

His Phe Ser Tyr Gly Asp Gly Asn Leu Ala Pro Asn Thr Ala Asn Val  
1315 1320 1325

Gln Met Thr Phe Leu Arg Leu Leu Ser Thr Glu Gly Ser Gln Asn Ile  
1330 1335 1340

Thr Tyr His Cys Lys Asn Ser Ile Ala Tyr Leu Asp Glu Ala Ala Gly  
1345 1350 1355 1360

15

Asn Leu Lys Lys Ala Leu Leu Ile Gln Gly Ser Asn Asp Val Glu Met  
1365 1370 1375

Arg Ala Glu Gly Asn Ser Arg Phe Thr Tyr Thr Ala Leu Lys Asp Gly  
1380 1385 1390

20

Cys Thr Lys His Thr Gly Lys Trp Gly Lys Thr Val Ile Glu Tyr Arg  
1395 1400 1405

Ser Gln Lys Thr Ser Arg Leu Pro Ile Ile Asp Ile Ala Pro Met Asp  
1410 1415 1420

Ile Gly Gly Ala Glu Gln Glu Phe Gly Val Asp Ile Gly Pro Val Cys  
1425 1430 1435 1440

Phe Leu

10 (2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 267 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

20

-209-

ATAGGCCCTT TGGAGACGGC TGTTTTCCAG ACTCCAAACT ATCGTGTAC ACGTGTGGGA 60  
AATGAAGTGT CTTTCAATTG TGAGCAAACC CTGGACCACA ATACTATGTA CTGGTACAAG 120  
CAAGACTCTA AGAAATTGCT GAAGATTATG TTTAGCTACA ATAATAAGCA ACTCATTTGA 180  
AACGAAACAG TTCCAAGGCG CTTCTCACCT CAGTCTTCAG ATAAAGCTCA TTTGAATCCT 240  
CGAATCAAGT CTGTAGAGCT GGAGGAC 267

10

(2) INFORMATION FOR SEQ ID NO:14:

## (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 54 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(11) MOLECULE TYPE: peptide

20

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:14:

-210-

Gly Pro Ser Gly Asp Gln Gly Ala Ser Gly Pro Ala Gly Pro Ser Gly  
1 5 10 15

Pro Arg Gly Pro Pro Gly Pro Val Gly Pro Ser Gly Lys Asp Gly Ala  
20 25 30

Asn Gly Ile Pro Gly Pro Ile Gly Pro Pro Gly Pro Arg Gly Arg Ser  
35 40 45

10 Gly Glu Thr Gly Pro Ala  
50

(2) INFORMATION FOR SEQ ID NO:15:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 731 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: DNA (genomic)



-211-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

	AGAATATAGA TAGATATGTC TGTGCTGACC GTGGCCTTTT GCCTCTTCCT TCTACACAGG	60
5	GTCCTTCTGG AGACCAAGGT GCTTCTGGTC CTGCTGGTCC TTCTGGCCCT AGAGTAAGTG	120
	ACATGGAGTT GGAAGATGGA GGGGGCCCTT CAGAGAGTGT GGGCCTGTGT TCCCATGGGG	180
	AGGGAATGCG TGCTGCTTCT GGGGAAGCTG TGGGCTCAGG GGTCTCACT CAGTAATGGG	240
10	GGCAGGACTG GCTCATGTGC CTATGGCCAG AAAAGCGCCT GAGGCCACAA TGGCTGTAAG	300
	ACAAACATGA ATCAGCCTCT CGCTGTCAGA CAGAACAGCA TTTTACAAAG AGGAGCTTAG	360
15	GAGGGTAGGC AAGCCATGGA GCTATCCTGC TGGTTCTTGG CCAAATAGAG ACCAACTTAG	420
	GGTTCCATGA CTGAGCATGT GAAGAACTGG GGGCGGAGTG GCTGGTGCTA TCAGGACAGC	480
	CACCTACCCA GCCCCAGCGA CTCCCCAGCC TTCCCTGTGG TGACCACTCT TTCTTCACGA	540
20	CCTCTCTCTC TTGCAGGGTC CTCCTGGCCC CGTCGGTCCC TCTGGCAAAG ATGGTGCTAA	600
	TGGAATCCCT GGCCCCATG GGCCTCCTGG TCCCCGTGGA CGATCAGGCG AAACCGGCC	660

-212-

TGCTGTAAGT GTCCTGACTC CTTCCCTGCT GTCGAGGTGT CCCTACCATC CGGGAGGCTT 720

GAGCTCTTTT T 731

5 (2) INFORMATION FOR SEQ ID NO:16:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: protein

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

20 Gly Glu Ser Val Ala Ser Lys His Ala Ile Tyr Ala Val Cys  
1 5 10

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5502 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "DNA"

10

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..5502

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

48

ATG GAG AGC ACC TCC CCG CGA GGT CTC CGG TGC CCA CAG CTC TGC AGC

Met Glu Ser Thr Ser Pro Arg Gly Leu Arg Cys Pro Gln Leu Cys Ser

1265

1260

1255

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-214-

96 CAC TCT GGC GCC ATG AGA GCG CCG ACC ACC GCT CGC TGC TCC GGA TGC  
 His Ser Gly Ala Met Arg Ala Pro Thr Thr Ala Arg Cys Ser Gly Cys  
 1270 1275 1280

144 ATC CAA CCG GTG CGT TGG AGG GGC TTC CTG CCA CTT GTC CTG GCT GTC  
 Ile Gln Arg Val Arg Trp Arg Gly Phe Leu Pro Leu Val Leu Ala Val  
 1285 1290 1295

192 TTG ATG GGG ACA AGT CAT GCC CAA CCG GAT TCC ATA GGG AGA TAC GAA  
 Leu Met Gly Thr Ser His Ala Gln Arg Asp Ser Ile Gly Arg Tyr Glu  
 1300 1305 1310 1315

240 CCA GCT AGC AGG GAT GCG AAT CCG TTG TGG CAC CCC GTG GGC AGC CAC  
 Pro Ala Ser Arg Asp Ala Asn Arg Leu Trp His Pro Val Gly Ser His  
 1320 1325 1330

288 CCC GCA GCG GCT GCA GCC AAG GTG TAC AGT CTG TTC CGA GAG CCT GAC  
 Pro Ala Ala Ala Ala Lys Val Tyr Ser Leu Phe Arg Glu Pro Asp  
 1335 1340 1345

336 GCG CCG GTC CCC GGC TTG TCG CCC TCT GAG TGG AAC CAG CCG GCC CAG  
 Ala Pro Val Pro Gly Leu Ser Pro Ser Glu Trp Asn Gln Pro Ala Gln  
 1350 1355 1360

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 10  
 15  
 20

-215-

384

GGG AAC CCG GGA TGG CTC GCA GAG GCC GAG GCC AGG AGG CCA CCT CGA  
 Gly Asn Pro Gly Trp Leu Ala Glu Ala Glu Ala Arg Arg Pro Pro Arg  
 1365 1370 1375

432

5 ACC CAG CAG CTG CGT CGA GTC CAG CCA CCT GTC CAG ACT CGG AGA AGC  
 Thr Gln Gln Leu Arg Arg Val Gln Pro Pro Val Gln Thr Arg Arg Ser  
 1380 1385 1390 1395

480

CAT CCC CGG GGC CAG CAG CAG ATA GCA GCC CGG GCT GCA CCT TCT GTC  
 10 His Pro Arg Gly Gln Gln Gln Ile Ala Ala Arg Ala Ala Pro Ser Val  
 1400 1405 1410

528

GCG CGC CTG GAA ACC CCT CAG CAG CGA CCC GCG GCT GCA CGG CGA GGG CGG  
 Ala Arg Leu Glu Thr Pro Gln Arg Pro Ala Ala Ala Arg Arg Gly Arg  
 1415 1420 1425

15

576

CTC ACT GGG AGA AAT GTC TGC GGG GGA CAG TGC TGC CCA GGA TGG ACA  
 Leu Thr Gly Arg Asn Val Cys Gly Gly Gln Cys Cys Pro Gly Trp Thr  
 1430 1435 1440

20

624

ACA TCA AAC AGC ACC AAC CAC TGT ATC AAA CCT GTG TGT CAG CCT CCC  
 Thr Ser Asn Ser Thr Asn His Cys Ile Lys Pro Val Cys Gln Pro Pro  
 1445 1450 1455

-216-

672 TGT CAG AAC CGA GGC TCC TGC AGC AGG CCC CAG GTC TGC ATC TGC CGT  
 Cys Gln Asn Arg Gly Ser Cys Ser Arg Pro Gln Val Cys Ile Cys Arg  
 1460 1465 1470 1475  
  
 720 TCT GGC TTC CGT GGG GCG CGC TGT GAG GAG GTC ATC CCT GAG GAG GAA  
 Ser Gly Phe Arg Gly Ala Arg Cys Glu Glu Val Ile Pro Glu Glu Glu  
 1480 1485 1490  
  
 768 TTT GAC CCT CAG AAT GCC AGG CCT GTG CCC AGA CGC TCA GTG GAG AGA  
 Phe Asp Pro Gln Asn Ala Arg Pro Val Pro Arg Arg Ser Val Glu Arg  
 1495 1500 1505  
  
 816 GCA CCC GGT CCT CAC AGA AGC AGT GAG GCC AGA GGA AGT CTA GTG ACC  
 Ala Pro Gly Pro His Arg Ser Ser Glu Ala Arg Gly Ser Leu Val Thr  
 1510 1515 1520  
  
 864 AGA ATA CAG CCG CTG GTA CCA CCA CCA TCA CCA CCT CCA TCT CGG CGC  
 Arg Ile Gln Pro Leu Val Pro Pro Pro Ser Pro Pro Pro Ser Arg Arg  
 1525 1530 1535  
  
 912 CTC AGC CAG CCC TGG CCC CTG CAG CAG CAC TCA GGG CCG TCC AGG ACA  
 Leu Ser Gln Pro Trp Pro Leu Gln His Ser Gly Pro Ser Arg Thr  
 1540 1545 1550 1555

-217-

5 GGT CGT CGG TAT CCG GCC ACT GGT GCC AAT GGC CAG CTG ATG TCC AAC 960  
Val Arg Arg Tyr Pro Ala Thr Gly Ala Asn Gly Gln Leu Met Ser Asn  
1560 1565 1570

5 GCT TTG CCT TCA GGA CTC GAG CTG AGA GAC AGC AGC CCA CAG GCA GCA 1008  
Ala Leu Pro Ser Gly Leu Glu Leu Arg Asp Ser Ser Pro Gln Ala Ala  
1575 1580 1585

10 CAT GTG AAC CAT CTC TCA CCC CCC TGG GGG CTG AAC CTC ACC GAG AAA 1056  
His Val Asn His Leu Ser Pro Pro Trp Gly Leu Asn Leu Thr Glu Lys  
1590 1595 1600

15 ATC AAG AAA ATC AAA GTC GTC TTC ACC CCC ACC ATC TGC AAG CAG ACC 1104  
Ile Lys Lys Ile Lys Val Val Phe Thr Pro Thr Ile Cys Lys Gln Thr  
1605 1610 1615

15 TGT GCC CGG GGA CGC TGT GCC AAC AGC TGT GAG AAG GGT GAC ACC ACC 1152  
Cys Ala Arg Gly Arg Cys Ala Asn Ser Cys Glu Lys Gly Asp Thr Thr  
1620 1625 1630 1635

20 ACC TTG TAC AGT CAG GGT GGC CAT GGG CAT GAC CCC AAG TCT GGC TTC 1200  
Thr Leu Tyr Ser Gln Gly Gly His Gly His Asp Pro Lys Ser Gly Phe  
1640 1645 1650

-218-

	CGT ATC TAT TTC TGC CAA ATC CCC TGC CTG AAT GGT GGC CGC TGC ATC	1248
	Arg Ile Tyr Phe Cys Gln Ile Pro Cys Leu Asn Gly Gly Arg Cys Ile	
	1655 1660 1665	
5	GGC CGG GAC GAG TGC TGG TGT CCA GCC AAC TCC ACA GGA AAG TTC TGC	1296
	Gly Arg Asp Glu Cys Trp Cys Pro Ala Asn Ser Thr Gly Lys Phe Cys	
	1670 1675 1680	
	CAT CTG CCT GTC CCG CAG CCA GAC AGG GAA CCT GCA GGG CGA GGT TCC	1344
10	His Leu Pro Val Pro Gln Pro Asp Arg Glu Pro Ala Gly Arg Gly Ser	
	1685 1690 1695	
	CGG CAC AGA ACC CTG CTG GAA GGT CCC CTG AAG CAA TCC ACC TTC ACG	1392
	Arg His Arg Thr Leu Leu Glu Gly Pro Leu Lys Gln Ser Thr Phe Thr	
15	1700 1705 1710 1715	
	CTG CCT CTC TCT AAC CAG CAG CTC GCC TCT GTG AAC CCC TCG CTG GTG AAG	1440
	Leu Pro Leu Ser Asn Gln Leu Ala Ser Val Asn Pro Ser Leu Val Lys	
	1720 1725 1730	
20	GTG CAA ATT CAT CAC CCG CCT GAG GCC TCT GTG CAG ATT CAC CAG GTG	1488
	Val Gln Ile His His Pro Pro Glu Ala Ser Val Gln Ile His Gln Val	
	1735 1740 1745	



-219-

1536

GCC CGG GTC CGG GGT GAG CTG GAC CCC GTG CTG GAG GAC AAC AGT GTG  
Ala Arg Val Arg Gly Glu Leu Asp Pro Val Leu Glu Asp Asn Ser Val  
1750 1755 1760

1584

GAG ACC AGA GCC TCT CAT CGC CCC CAC GGC AAC CTA GGC CAC AGC CCC  
Glu Thr Arg Ala Ser His Arg Pro His Gly Asn Leu Gly His Ser Pro  
1765 1770 1775

1632

TGG GCC AGC AAC AGC ATA CCC GCT CGG GCC GGA GAG GCC CCT CGG CCA  
Trp Ala Ser Asn Ser Ile Pro Ala Arg Ala Gly Glu Ala Pro Arg Pro  
1780 1785 1790 1795

1680

CCA CCA GTG CTG TCT AGG CAT TAT GGA CTT CTG GGC CAG TGT TAC CTG  
Pro Pro Val Leu Ser Arg His Tyr Gly Leu Leu Gly Gln Cys Tyr Leu  
1800 1805 1810

15

1728

AGC ACG GTG AAT GGA CAG TGT GCT AAC CCC CTA GGT AGT CTG ACT TCT  
Ser Thr Val Asn Gly Gln Cys Ala Asn Pro Leu Gly Ser Leu Thr Ser  
1815 1820 1825

20

1776

CAG GAG GAC TGC TGT GGC AGT GTG GGG ACC TTC TGG GGG GTG ACC TCC  
Gln Glu Asp Cys Cys Gly Ser Val Gly Thr Phe Trp Gly Val Thr Ser  
1830 1835 1840

-220-

1824  
 TGT GCT CCC TGC CCA CCC AGA CAA GAG GGT CCA GCC TTC CCA GTG ATT  
 Cys Ala Pro Cys Pro Pro Arg Gln Glu Gly Pro Ala Phe Pro Val Ile  
 1845 1850 1855

5 1872  
 GAA AAT GGC CAG CTG GAG TGT CCC CAA GGA TAC AAG AGA CTG AAC CTC  
 Glu Asn Gly Gln Leu Glu Cys Pro Gln Gly Tyr Lys Arg Leu Asn Leu  
 1860 1865 1870 1875

10 1920  
 AGC CAC TGC CAA GAT ATC AAT GAG TGC CTG ACC CTG GGC CTC TGC AAG  
 Ser His Cys Gln Asp Ile Asn Glu Cys Leu Thr Leu Gly Leu Cys Lys  
 1880 1885 1890

15 1968  
 GAC TCG GAG TGC GTG AAC ACC AGG GGC AGC TAC CTG TGC ACC TGC AGG  
 Asp Ser Glu Cys Val Asn Thr Arg Gly Ser Tyr Leu Cys Thr Cys Arg  
 1895 1900 1905

20 2016  
 CCT GGC CTC ATG CTG GAT CCG TCA AGG AGC CGC TGC GTA TCG GAC AAG  
 Pro Gly Leu Met Leu Asp Pro Ser Arg Ser Arg Cys Val Ser Asp Lys  
 1910 1915 1920

2064  
 GCT GTC TCC ATG CAG CAG GGA CTA TGC TAC CGG TCA CTG GGG TCT GGT  
 Ala Val Ser Met Gln Gln Gly Leu Cys Tyr Arg Ser Leu Gly Ser Gly  
 1925 1930 1935

-221-

2112

ACC TGC ACC CTG CCT TTG GTT CAT CGG ATC ACC AAG CAG ATA TGC TGC  
 Thr Cys Thr Leu Pro Leu Val His Arg Ile Thr Lys Gln Ile Cys Cys  
 1940 1945 1950 1955

2160

5 TGC AGC CGT GTG GGC AAA GCC TGG GGT AGC ACA TGT GAA CAG TGT CCC  
 Cys Ser Arg Val Gly Lys Ala Trp Gly Ser Thr Cys Glu Gln Cys Pro  
 1960 1965 1970

2208

10 CTG CCT GGC ACA GAA GCC TTC AGG GAG ATC TGC CCT GCT GGC CAT GGC  
 Leu Pro Gly Thr Glu Ala Phe Arg Glu Ile Cys Pro Ala Gly His Gly  
 1975 1980 1985

2256

15 TAC ACC TAC TCG AGC TCA GAC ATC CGC CTG TCT ATG AGG AAA GCC GAA  
 Tyr Thr Tyr Ser Ser Ser Asp Ile Arg Leu Ser Met Arg Lys Ala Glu  
 1990 1995 2000

2304

GAA GAG GAA CTG GCT AGC CCC TTA AGG GAG CAG ACA GAG CAG AGC ACT  
 Glu Glu Glu Leu Ala Ser Pro Leu Arg Glu Gln Thr Glu Gln Ser Thr  
 2005 2010 2015

2352

20 GCA CCC CCA CCT GGG CAA GCA GAG AGG CAA CCA CTC CGG GCA GCC ACC  
 Ala Pro Pro Pro Gly Gln Ala Glu Arg Gln Pro Leu Arg Ala Ala Thr  
 2020 2025 2030 2035

-222-

2400  
GCC ACC TGG ATT GAG GCT GAG ACC CTC CCT GAC AAA GGT GAC TCT CGG  
Ala Thr Trp Ile Glu Ala Glu Thr Leu Pro Asp Lys Gly Asp Ser Arg  
2040 2045 2050

2448  
5. GCT GTT CAG ATC ACA ACC AGT GCT CCC CAC CTA CCT GCC CGG GTA CCA  
Ala Val Gln Ile Thr Thr Ser Ala Pro His Leu Pro Ala Arg Val Pro  
2055 2060 2065

2496  
10 GGG GAT GCC ACT GGA AGA CCA GCA CCA TCC TTG CCT GGA CAG GGC ATT  
Gly Asp Ala Thr Gly Arg Pro Ala Pro Ser Leu Pro Gly Gln Gly Ile  
2070 2075 2080

2544  
CCA GAG AGT CCA GCA GAA GAG CAA GTG ATT CCC TCC AGT GAT GTC TTG  
Pro Glu Ser Pro Ala Glu Glu Gln Val Ile Pro Ser Ser Asp Val Leu  
2085 2090 2095

2592  
GTG ACA CAC AGC CCC CCA GAC TTT GAT CCA TGT TTT GCT GGA GCC TCC  
Val Thr His Ser Pro Pro Asp Phe Asp Pro Cys Phe Ala Gly Ala Ser  
2100 2105 2110 2115

2640  
20 AAC ATC TGT GGC CCT GGG ACC TGT GTG AGC CTC CCA AAT GGA TAC AGA  
Asn Ile Cys Gly Pro Gly Thr Cys Val Ser Leu Pro Asn Gly Tyr Arg  
2120 2125 2130

-223-

2688

TGT GTC TGC AGC CCT GGC TAC CAG CTA CAC CCC AGC CAA GAC TAC TGT  
Cys Val Cys Ser Pro Gly Tyr Gln Leu His Pro Ser Gln Asp Tyr Cys  
2135 2140 2145

2736

ACT GAT GAC AAC GAG TGT ATG AGG AAC CCC TGT GAA GGA AGA GGG CGC  
Thr Asp Asp Asn Asn Glu Cys Met Arg Asn Pro Cys Glu Gly Arg Gly Arg  
2150 2155 2160

2784

TGT GTC AAC AGT GTG GGC TCC TAC TCC TGC CTC TGC TAT CCT GGC TAC  
Cys Val Asn Ser Val Gly Ser Tyr Ser Cys Leu Cys Tyr Pro Gly Tyr  
2165 2170 2175

2832

ACA CTA GTC ACC CTC GGA GAC ACA CAG GAG TGC CAA GAT ATC GAT GAG  
Thr Leu Val Thr Leu Gly Asp Thr Gln Glu Cys Gln Asp Ile Asp Glu  
2180 2185 2190 2195

2880

TGT GAG CAG CCC GGG GTG TGC AGT GGT GGG CGA TGC AGC AAC ACG GAG  
Cys Glu Gln Pro Gly Val Cys Ser Gly Gly Arg Cys Ser Asn Thr Glu  
2200 2205 2210

2928

GGC TCG TAC CAC TGC GAG TGT GAT CGG GGC TAC ATC ATG GTC AGG AAA  
Gly Ser Tyr His Cys Glu Cys Asp Arg Gly Tyr Ile Met Val Arg Lys  
2215 2220 2225

20

5

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15

- 224 -

2976

GGA CAC TGT CAA GAT ATC AAC GAA TGC CGT CAC CCT GGT ACC TGC CCT  
Gly His Cys Gln Asp Ile Asn Glu Cys Arg His Pro Gly Thr Cys Pro  
2230 2235 2240

3024

GAT GGG AGA TGC GTC AAC TCC CCT GGC TCC TAC ACT TGT CTG GCC TGT  
Asp Gly Arg Cys Val Asn Ser Pro Gly Ser Tyr Thr Cys Leu Ala Cys  
2245 2250 2255

3072

GAG GAG GGC TAT GTA GGC CAG AGT GGG AGC TGT GTA GAT GTC AAT GAG  
Glu Glu Gly Tyr Val Gly Gln Ser Gly Ser Cys Val Asp Val Asn Glu  
2260 2265 2270 2275

3120

TGT CTG ACC CCT GGG ATA TGT ACC CAT GGA AGG TGC ATC AAC ATG GAA  
Cys Leu Thr Pro Gly Ile Cys Thr His Gly Arg Cys Ile Asn Met Glu  
2280 2285 2290

3168

GGC TCC TTT AGA TGC TCC TGT GAG CCG GGC TAT GAG GTC ACC CCA GAC  
Gly Ser Phe Arg Cys Ser Cys Glu Pro Gly Tyr Glu Val Thr Pro Asp  
2295 2300 2305

3216

AAG AAG GGC TGC CGA GAT GTG GAC GAG TGT GCC AGC CGA GCC TCG TGC  
Lys Lys Gly Cys Arg Asp Val Asp Glu Cys Ala Ser Arg Ala Ser Cys  
2310 2315 2320

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15

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- 225 -

3264

CCC ACG GGC CTC TGC CTC AAC ACG GAG GGC TCC TTC ACC TGC TCA GCC  
 Pro Thr Gly Leu Cys Leu Asn Thr Glu Gly Ser Phe Thr Cys Ser Ala  
 2325 2330 2335

3312

5 TGT CAG AGC GGG TAC TGG GTG AAC GAA GAT GGC ACT GCC TGT GAA GAC  
 Cys Gln Ser Gly Tyr Trp Val Asn Glu Asp Gly Thr Ala Cys Glu Asp  
 2340 2345 2350 2355

3360

TTG GAT GAA TGT GCC TTC CCT GGA GTC TGC CCC ACA GGC GTC TGC ACC  
 10 Leu Asp Glu Cys Ala Phe Pro Gly Val Cys Pro Thr Gly Val Cys Thr  
 2360 2365 2370

3408

AAT ACT GTA GGC TCC TTC TCC TGC AAG GAC TGT GAC CAG GGC TAC CGG  
 Asn Thr Val Gly Ser Phe Ser Cys Lys Asp Cys Asp Gln Gly Tyr Arg  
 2375 2380 2385

3456

CCC AAC CCC CTG GGC AAC AGA TGC GAA GAT GTG GAT GAG TGT GAA GGT  
 Pro Asn Pro Leu Gly Asn Arg Cys Glu Asp Val Asp Glu Cys Glu Gly  
 2390 2395 2400

3504

CCC CAA AGC AGC TGC CGG GGA GGC GAA TGC AAG AAC ACA GAA GGT TCC  
 Pro Gln Ser Ser Cys Arg Gly Gly Glu Cys Lys Asn Thr Glu Gly Ser  
 2405 2410 2415

20

15

-226-

3552  
TAC CAA TGC CTC TGT CAC CAG GGC TTC CAG CTG GTC AAT GGC ACC ATG  
Tyr Gln Cys Leu Cys His Gln Gly Phe Gln Leu Val Asn Gly Thr Met  
2420 2425 2430 2435

3600  
5 TGT GAG GAC GTG AAT GAG TGT GTT GGG GAA GAG CAT TGT GCT CCT CAC  
Cys Glu Asp Val Asn Glu Cys Val Gly Glu His Cys Ala Pro His  
2440 2445 2450

3648  
10 GGC GAG TGC CTC AAC AGC CTG GGC TCC TTC TTC TGC CTC TGT GCA CCC  
Gly Glu Cys Leu Asn Ser Leu Gly Ser Phe Phe Cys Leu Cys Ala Pro  
2455 2460 2465

3696  
15 GGC TTT GCT AGT GCT GAG GGC GGC ACC AGA TGC CAG GAT GTT GAT GAA  
Gly Phe Ala Ser Ala Glu Gly Gly Thr Arg Cys Gln Asp Val Asp Glu  
2470 2475 2480

3744  
TGT GCA GCC ACA GAC CCG TGT CCG GGA GGA CAC TGT GTC AAC ACA GAG  
Cys Ala Ala Thr Asp Pro Cys Pro Gly Gly His Cys Val Asn Thr Glu  
2485 2490 2495

3792  
20 GGC TCC TTC AGC TGT CTG TGT GAG ACT GCT TCC TTC CAG CCC TCC CCA  
Gly Ser Phe Ser Cys Leu Cys Glu Thr Ala Ser Phe Gln Pro Ser Pro  
2500 2505 2510 2515



-227-

3840

GAC AGC GGA GAA TGT TTG GAT ATT GAT GAG TGT GAG GAC CGT GAA GAC  
 Asp Ser Gly Glu Cys Leu Asp Ile Asp Glu Cys Glu Asp Arg Glu Asp  
 2520 2525 2530

3888

5 CCG GTG TGC GGA GCC TGG AGG TGT GAG AAC AGT CCT GGT TCC TAC CGC  
 Pro Val Cys Gly Ala Trp Arg Cys Glu Asn Ser Pro Gly Ser Tyr Arg  
 2535 2540 2545

3936

TGC ATC CTG GAC TGC CAG CCT GGA TTC TAT GTG GCG CCA AAT GGA GAC  
 10 Cys Ile Leu Asp Cys Gln Pro Gly Phe Tyr Val Ala Pro Asn Gly Asp  
 2550 2555 2560

3984

TGC ATT GAC ATA GAT GAA TGT GCC AAT GAC ACT GTG TGT GGG AAC CAT  
 Cys Ile Asp Ile Asp Glu Cys Ala Asn Asp Thr Val Cys Gly Asn His  
 15 2565 2570 2575

4032

GGC TTC TGT GAC AAC ACG GAC GGC TCC TTC CGC TGC CTG TGT GAC CAG  
 Gly Phe Cys Asp Asn Thr Asp Gly Ser Phe Arg Cys Leu Cys Asp Gln  
 2580 2585 2590 2595

20 GGC TTC GAG ACC TCA CCA GGC TGG GAG TGT GTT GAT GTG AAC GAG  
 Gly Phe Glu Thr Ser Pro Ser Gly Trp Glu Cys Val Asp Val Asn Glu  
 2600 2605 2610

4080

- 228 -

4128

TGT GAG CTC ATG ATG GCA GTG TGT GGG GAT GCG CTC TGT GAG AAC GTG  
 Cys Glu Leu Met Met Ala Val Cys Gly Asp Ala Leu Cys Glu Asn Val  
 2615 2620 2625

4176

5 GAA GGC TCC TTC CTG TGC CTT TGC GCC AGT GAC CTT GAG GAG TAC GAC  
 Glu Gly Ser Phe Leu Cys Leu Cys Ala Ser Asp Leu Glu Glu Tyr Asp  
 2630 2635 2640

4224

GCA GAA GAA GGA CAC TGC TGC CGT CCT CGG GTG GCT GGA GCT CAG AGA ATC  
 10 Ala Glu Glu Gly His Cys Arg Pro Arg Val Ala Gly Ala Gln Arg Ile  
 2645 2650 2655

4272

CCA GAG GTC CGG ACA GAG GAC CAG GCT CCA AGC CTT ATC CGC ATG GAA  
 Pro Glu Val Arg Thr Glu Asp Gln Ala Pro Ser Leu Ile Arg Met Glu  
 15 2660 2665 2670 2675

4320

TGC TAC TCT GAA CAC AAT GGT GGT CCT CCC TGC TCT CAA ATC CTG GGC  
 Cys Tyr Ser Glu His Asn Gly Gly Pro Pro Cys Ser Gln Ile Leu Gly  
 2680 2685 2690

20

CAG AAC TCC ACA CAG GCC GAG TGC TGC TGC ACT CAG GGT GCC AGA TGG  
 Gln Asn Ser Thr Gln Ala Glu Cys Cys Cys Thr Gln Gly Ala Arg Trp  
 2695 2700 2705

4368

-229-

4416

GGA AAG GCC TGT GCG CCC TGC CCA TCT GAG GAC TCA GTT GAA TTC AGT  
 Gly Lys Ala Cys Ala Pro Cys Pro Ser Glu Asp Ser Val Glu Phe Ser  
 2710 2715 2720

4464

5 CAG CTC TGC CCC AGT GGT CAA GGT TAC ATC CCA GTG GAA GGA GCC TGG  
 Gln Leu Cys Pro Ser Gly Gln Gly Tyr Ile Pro Val Glu Gly Ala Trp  
 2725 2730 2735

4512

ACA TTT GGA CAA ACC ATG TAT ACA GAT GCC GAT GAA TGT GTA CTG TTT  
 Thr Phe Gly Gln Thr Met Tyr Thr Asp Ala Asp Glu Cys Val Leu Phe  
 2740 2745 2750 2755

4560

GGG CCT GCT CTC TGC CAG AAT GGC CGA TGC TCA AAC ATA GTG CCT GGC  
 Gly Pro Ala Leu Cys Cys Gln Asn Gly Arg Cys Ser Asn Ile Val Pro Gly  
 2760 2765 2770

4608

TAC ATT TGC CTG TGC AAC CCT GGC TAC CAC TAT GAT GCC TCC AGC AGG  
 Tyr Ile Cys Leu Cys Asn Pro Gly Tyr His Tyr Asp Ala Ser Ser Arg  
 2775 2780 2785

4656

20 AAG TGC CAG GAT CAC AAC GAA TGC CAG GAC TTG GCC TGT GAG AAC GGT  
 Lys Cys Gln Asp His Asn Glu Cys Gln Asp Leu Ala Cys Glu Asn Gly  
 2790 2795 2800

- 230 -

4704

GAG TGT GTG AAC CAA GAA GGC TCC TTC CAT TGC CTC TGC AAT CCC CCC  
Glu Cys Val Asn Gln Glu Gly Ser Phe His Cys Leu Cys Asn Pro Pro  
2805 2810 2815

4752

5 CTC ACC CTA GAC CTC AGT GGG CAG CGC TGT GTG AAC ACG ACC AGC AGC  
Leu Thr Leu Asp Leu Ser Gly Gln Arg Cys Val Asn Thr Thr Ser Ser  
2820 2825 2830 2835

4800

10 ACG GAG GAC TTC CCT GAC CAT GAC ATC CAC ATG GAC ATC TGC TGG AAA  
Thr Glu Asp Phe Pro Asp His Asp Ile His Met Asp Ile Cys Trp Lys  
2840 2845 2850

4848

15 AAA GTC ACC AAT GAT GTG TGC AGC CAG CCC TTG CGT GGG CAC CAT ACC  
Lys Val Thr Asn Asp Val Cys Ser Gln Pro Leu Arg Gly His His Thr  
2855 2860 2865

4896

ACC TAT ACA GAA TGC TGC TGC CAA GAT GGG GAG GCC TGG AGC CAG CAA  
Thr Tyr Thr Glu Cys Cys Gln Asp Gly Glu Ala Trp Ser Gln Gln  
2870 2875 2880

20 TGC GCT CTG TGC CCG CCC AGG AGC TCT GAG GTC TAC GCT CAG CTG TGC  
Cys Ala Leu Cys Pro Pro Arg Ser Ser Glu Val Tyr Ala Gln Leu Cys  
2885 2890 2895

4944

-231-

4992

AAC GTG GCT CGG ATT GAG GCA GAG CGC GGA GCA GGG ATC CAC TTC CGG  
 Asn Val Ala Arg Ile Glu Ala Glu Arg Gly Ala Gly Ile His Phe Arg  
 2900 2905 2910 2915

5040

CCA GGC TAT GAG TAT GGC CCT GGC CTG GAC GAT CTG CCT GAA AAC CTC  
 Pro Gly Tyr Glu Tyr Gly Pro Gly Leu Asp Asp Leu Pro Glu Asn Leu  
 2920 2925 2930

5088

TAC GGC CCA GAT GGG GCT CCC TTC TAT AAC TAC CTA GGC CCC GAG GAC  
 Tyr Gly Pro Asp Gly Ala Pro Phe Tyr Asn Tyr Leu Gly Pro Glu Asp  
 2935 2940 2945

5136

ACT GCC CCT GAG CCT CCC TTC TCC AAC CCA GCC AGC CAG CCG GGA GAC  
 Thr Ala Pro Glu Pro Pro Phe Ser Asn Pro Ala Ser Gln Pro Gly Asp  
 2950 2955 2960

5184

AAC ACA CCT GTC CTT GAG CCT CCT CTG CAG CCC TCT GAA CTT CAG CCT  
 Asn Thr Pro Val Leu Glu Pro Pro Leu Gln Pro Ser Glu Leu Gln Pro  
 2965 2970 2975

5232

CAC TAT CTA GCC AGC CAC TCA GAA CCC CCT GCC TCC TTC GAA GGC CTT  
 His Tyr Leu Ala Ser His Ser Glu Pro Pro Ala Ser Phe Glu Gly Leu  
 2980 2985 2990 2995

20

-232-

5280  
CAG GCT GAG GAA TGT GGC ATC CTG AAT GGC TGT GAG AAT GGC CGC TGC  
Gln Ala Glu Glu Cys Gly Ile Leu Asn Gly Cys Glu Asn Gly Arg Cys  
3000 3005 3010

5328  
GTG CGT GTG CGG GAG GGC TAC ACT TGC GAC TGC TTT GAG GGC TTC CAG  
Val Arg Val Arg Glu Gly Tyr Thr Cys Asp Cys Phe Glu Gly Phe Gln  
3015 3020 3025

5376  
CTG GAT GCG CCC ACA TTG GCC TGT GTG GAT GTG AAC GAG TGT GAA GAC  
Leu Asp Ala Pro Thr Leu Ala Cys Val Asp Val Asn Glu Cys Glu Asp  
3030 3035 3040

5424  
TTG AAC GGG CCT GCA CGA CTC TGT GCA CAC GGT CAC TGT GAG AAC ACA  
Leu Asn Gly Pro Ala Arg Leu Cys Ala His Gly His Cys Glu Asn Thr  
3045 3050 3055

5472  
GAG GGT TCC TAT CGC TGC CAC TGT TCG CCA GGT TAC GTG GCA GAG CCA  
Glu Gly Ser Tyr Arg Cys His Cys Ser Pro Gly Tyr Val Ala Glu Pro  
3060 3065 3070 3075

5502  
GGC CCC CCA CAC TGT GCG GCC AAG GAG TAG  
Gly Pro Pro His Cys Ala Ala Lys Glu \*  
3080 3085

20

-233-

## (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1834 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met Glu Ser Thr Ser Pro Arg Gly Leu Arg Cys Pro Gln Leu Cys Ser  
1 5 10 15  
His Ser Gly Ala Met Arg Ala Pro Thr Thr Ala Arg Cys Ser Gly Cys  
20 25 30  
Ile Gln Arg Val Arg Trp Arg Gly Phe Leu Pro Leu Val Leu Ala Val  
35 40 45  
Leu Met Gly Thr Ser His Ala Gln Arg Asp Ser Ile Gly Arg Tyr Glu  
50 55 60

Pro	Ala	Ser	Arg	Asp	Ala	Asn	Arg	Leu	Trp	His	Pro	Val	Gly	Ser	His
65					70				75					80	
Pro	Ala	Ala	Ala	Ala	Ala	Lys	Val	Tyr	Ser	Leu	Phe	Arg	Glu	Pro	Asp
					85				90					95	
Ala	Pro	Val	Pro	Gly	Leu	Ser	Pro	Ser	Glu	Trp	Asn	Gln	Pro	Ala	Gln
				100					105				110		
Gly	Asn	Pro	Gly	Trp	Leu	Ala	Glu	Ala	Glu	Ala	Arg	Arg	Pro	Pro	Arg
		115					120					125			
Thr	Gln	Gln	Leu	Arg	Arg	Val	Gln	Pro	Pro	Val	Gln	Thr	Arg	Arg	Ser
		130				135					140				
His	Pro	Arg	Gly	Gln	Gln	Gln	Ile	Ala	Ala	Arg	Ala	Ala	Pro	Ser	Val
145					150				155					160	
Ala	Arg	Leu	Glu	Thr	Pro	Gln	Arg	Pro	Ala	Ala	Ala	Arg	Arg	Gly	Arg
				165					170					175	
Leu	Thr	Gly	Arg	Asn	Val	Cys	Gly	Gly	Gln	Cys	Pro	Gly	Trp	Thr	
				180					185				190		



-235-

Thr Ser Asn Ser Thr Asn His Cys Ile Lys Pro Val Cys Gln Pro Pro  
 195 200 205  
 Cys Gln Asn Arg Gly Ser Cys Ser Arg Pro Gln Val Cys Ile Cys Arg  
 210 215 220  
 Ser Gly Phe Arg Gly Ala Arg Cys Glu Glu Val Ile Pro Glu Glu Glu  
 225 230 235 240  
 Phe Asp Pro Gln Asn Ala Arg Pro Val Pro Arg Arg Ser Val Glu Arg  
 245 250 255  
 Ala Pro Gly Pro His Arg Ser Ser Glu Ala Arg Gly Ser Leu Val Thr  
 260 265 270  
 Arg Ile Gln Pro Leu Val Pro Pro Pro Ser Pro Pro Ser Arg Arg  
 275 280 285  
 Leu Ser Gln Pro Trp Pro Leu Gln Gln His Ser Gly Pro Ser Arg Thr  
 290 295 300  
 Val Arg Arg Tyr Pro Ala Thr Gly Ala Asn Gly Gln Leu Met Ser Asn  
 305 310 315 320

-236-

Ala Leu Pro Ser Gly Leu Glu Leu Arg Asp Ser Ser Pro Gln Ala Ala  
 325 330 335

His Val Asn His Leu Ser Pro Pro Trp Gly Leu Asn Leu Thr Glu Lys  
 340 345 350

Ile Lys Lys Ile Lys Val Val Phe Thr Pro Thr Ile Cys Lys Gln Thr  
 355 360 365

10 Cys Ala Arg Gly Arg Cys Ala Asn Ser Cys Glu Lys Gly Asp Thr Thr  
 370 375 380

Thr Leu Tyr Ser Gln Gly Gly His Gly His Asp Pro Lys Ser Gly Phe  
 385 390 395 400

15 Arg Ile Tyr Phe Cys Gln Ile Pro Cys Leu Asn Gly Gly Arg Cys Ile  
 405 410 415

Gly Arg Asp Glu Cys Trp Cys Pro Ala Asn Ser Thr Gly Lys Phe Cys  
 420 425 430

His Leu Pro Val Pro Gln Pro Asp Arg Glu Pro Ala Gly Arg Gly Ser  
 435 440 445

-237-

Arg His Arg Thr Leu Leu Glu Gly Pro Leu Lys Gln Ser Thr Phe Thr  
 450 455 460  
 5 Leu Pro Leu Ser Asn Gln Leu Ala Ser Val Asn Pro Ser Leu Val Lys  
 465 470 475 480  
 Val Gln Ile His His Pro Pro Glu Ala Ser Val Gln Ile His Gln Val  
 485 490 495  
 10 Ala Arg Val Arg Gly Glu Leu Asp Pro Val Leu Glu Asp Asn Ser Val  
 500 505 510  
 Glu Thr Arg Ala Ser His Arg Pro His Gly Asn Leu Gly His Ser Pro  
 515 520 525  
 15 Trp Ala Ser Asn Ser Ile Pro Ala Arg Ala Gly Glu Ala Pro Arg Pro  
 530 535 540  
 Pro Pro Val Leu Ser Arg His Tyr Gly Leu Leu Gly Gln Cys Tyr Leu  
 545 550 555 560  
 Ser Thr Val Asn Gly Gln Cys Ala Asn Pro Leu Gly Ser Leu Thr Ser  
 565 570 575

- 238 -

Gln Glu Asp Cys Cys Gly Ser Val Gly Thr Phe Trp Gly Val Thr Ser  
 580 585 590  
  
 Cys Ala Pro Cys Pro Pro Arg Gln Glu Gly Pro Ala Phe Pro Val Ile  
 595 600 605  
  
 Glu Asn Gly Gln Leu Glu Cys Pro Gln Gly Tyr Lys Arg Leu Asn Leu  
 610 615 620  
  
 Ser His Cys Gln Asp Ile Asn Glu Cys Leu Thr Leu Gly Leu Cys Lys  
 625 630 635 640  
  
 Asp Ser Glu Cys Val Asn Thr Arg Gly Ser Tyr Leu Cys Thr Cys Arg  
 645 650 655  
  
 Pro Gly Leu Met Leu Asp Pro Ser Arg Ser Arg Cys Val Ser Asp Lys  
 660 665 670  
  
 Ala Val Ser Met Gln Gln Gly Leu Cys Tyr Arg Ser Leu Gly Ser Gly  
 675 680 685  
  
 Thr Cys Thr Leu Pro Leu Val His Arg Ile Thr Lys Gln Ile Cys Cys  
 690 695 700

15

20

-239-

Cys Ser Arg Val Gly Lys Ala Trp Gly Ser Thr Cys Glu Gln Cys Pro  
705 710 715 720

Leu Pro Gly Thr Glu Ala Phe Arg Glu Ile Cys Pro Ala Gly His Gly  
725 730 735

Tyr Thr Tyr Ser Ser Ser Asp Ile Arg Leu Ser Met Arg Lys Ala Glu  
740 745 750

10 Glu Glu Glu Leu Ala Ser Pro Leu Arg Glu Gln Thr Glu Gln Ser Thr  
755 760 765

Ala Pro Pro Pro Gly Gln Ala Glu Arg Gln Pro Leu Arg Ala Ala Thr  
770 775 780

15 Ala Thr Trp Ile Glu Ala Glu Thr Leu Pro Asp Lys Gly Asp Ser Arg  
785 790 795 800

Ala Val Gln Ile Thr Thr Ser Ala Pro His Leu Pro Ala Arg Val Pro  
805 810 815

Gly Asp Ala Thr Gly Arg Pro Ala Pro Ser Leu Pro Gly Gln Gly Ile  
820 825 830

-240-

Pro Glu Ser Pro Ala Glu Glu Gln Val Ile Pro Ser Ser Asp Val Leu  
 835 840 845  
  
 Val Thr His Ser Pro Pro Asp Phe Asp Pro Cys Phe Ala Gly Ala Ser  
 5 850 855 860  
  
 Asn Ile Cys Gly Pro Gly Thr Cys Val Ser Leu Pro Asn Gly Tyr Arg  
 865 870 875 880  
  
 Cys Val Cys Ser Pro Gly Tyr Gln Leu His Pro Ser Gln Asp Tyr Cys  
 10 885 890 895  
  
 Thr Asp Asp Asn Glu Cys Met Arg Asn Pro Cys Glu Gly Arg Gly Arg  
 900 905 910  
  
 Cys Val Asn Ser Val Gly Ser Tyr Ser Cys Leu Cys Tyr Pro Gly Tyr  
 15 915 920 925  
  
 Thr Leu Val Thr Leu Gly Asp Thr Gln Glu Cys Gln Asp Ile Asp Glu  
 20 930 935 940  
  
 Cys Glu Gln Pro Gly Val Cys Ser Gly Gly Arg Cys Ser Asn Thr Glu  
 945 950 955 960

-241-

Gly Ser Tyr His Cys Glu Cys Asp Arg Gly Tyr Ile Met Val Arg Lys  
 965 970 975

Gly His Cys Gln Asp Ile Asn Glu Cys Arg His Pro Gly Thr Cys Pro  
 980 985 990

Asp Gly Arg Cys Val Asn Ser Pro Gly Ser Tyr Thr Cys Leu Ala Cys  
 995 1000 1005

Glu Glu Gly Tyr Val Gly Gln Ser Gly Ser Cys Val Asp Val Asn Glu  
 1010 1015 1020

Cys Leu Thr Pro Gly Ile Cys Thr His Gly Arg Cys Ile Asn Met Glu  
 1025 1030 1035 1040

Gly Ser Phe Arg Cys Ser Cys Glu Pro Gly Tyr Glu Val Thr Pro Asp  
 1045 1050 1055

Lys Lys Gly Cys Arg Asp Val Asp Glu Cys Ala Ser Arg Ala Ser Cys  
 1060 1065 1070

Pro Thr Gly Leu Cys Leu Asn Thr Glu Gly Ser Phe Thr Cys Ser Ala  
 1075 1080 1085

-242-

Cys Gln Ser Gly Tyr Trp Val Asn Glu Asp Gly Thr Ala Cys Glu Asp  
1090 1095 1100

Leu Asp Glu Cys Ala Phe Pro Gly Val Cys Pro Thr Gly Val Cys Thr  
1105 1110 1115 1120

Asn Thr Val Gly Ser Phe Ser Cys Lys Asp Cys Asp Gln Gly Tyr Arg  
1125 1130 1135

Pro Asn Pro Leu Gly Asn Arg Cys Glu Asp Val Asp Glu Cys Glu Gly  
1140 1145 1150

Pro Gln Ser Ser Cys Arg Gly Gly Glu Cys Lys Asn Thr Glu Gly Ser  
1155 1160 1165

Tyr Gln Cys Leu Cys His Gln Gly Phe Gln Leu Val Asn Gly Thr Met  
1170 1175 1180

Cys Glu Asp Val Asn Glu Cys Val Gly Glu Glu His Cys Ala Pro His  
1185 1190 1195 1200

Gly Glu Cys Leu Asn Ser Leu Gly Ser Phe Phe Cys Leu Cys Ala Pro  
1205 1210 1215

15

20



-243-

Gly Phe Ala Ser Ala Glu Gly Gly Thr Arg Cys Gln Asp Val Asp Glu  
1220 1225 1230

Cys Ala Ala Thr Asp Pro Cys Pro Gly Gly His Cys Val Asn Thr Glu  
1235 1240 1245

5

Gly Ser Phe Ser Cys Leu Cys Glu Thr Ala Ser Phe Gln Pro Ser Pro  
1250 1255 1260

Asp Ser Gly Glu Cys Leu Asp Ile Asp Glu Cys Glu Asp Arg Glu Asp  
1265 1270 1275 1280

Pro Val Cys Gly Ala Trp Arg Cys Glu Asn Ser Pro Gly Ser Tyr Arg  
1285 1290 1295

15

Cys Ile Leu Asp Cys Gln Pro Gly Phe Tyr Val Ala Pro Asn Gly Asp  
1300 1305 1310

Cys Ile Asp Ile Asp Glu Cys Ala Asn Asp Thr Val Cys Gly Asn His  
1315 1320 1325

20

Gly Phe Cys Asp Asn Thr Asp Gly Ser Phe Arg Cys Leu Cys Asp Gln  
1330 1335 1340

-244-

Gly Phe Glu Thr Ser Pro Ser Gly Trp Glu Cys Val Asp Val Asn Glu  
1345 1350 1355 1360

Cys Glu Leu Met Met Ala Val Cys Gly Asp Ala Leu Cys Glu Asn Val  
1365 1370 1375

5

Glu Gly Ser Phe Leu Cys Leu Cys Ala Ser Asp Leu Glu Glu Tyr Asp  
1380 1385 1390

10 Ala Glu Glu Gly His Cys Arg Pro Arg Val Ala Gly Ala Gln Arg Ile  
1395 1400 1405

Pro Glu Val Arg Thr Glu Asp Gln Ala Pro Ser Leu Ile Arg Met Glu  
1410 1415 1420

15

Cys Tyr Ser Glu His Asn Gly Gly Pro Pro Cys Ser Gln Ile Leu Gly  
1425 1430 1435 1440

Gln Asn Ser Thr Gln Ala Glu Cys Cys Thr Gln Gly Ala Arg Trp  
1445 1450 1455

20

Gly Lys Ala Cys Ala Pro Cys Pro Ser Glu Asp Ser Val Glu Phe Ser  
1460 1465 1470

-245-

Gln Leu Cys Pro Ser Gly Gln Gly Tyr Ile Pro Val Glu Gly Ala Trp  
 1475 1480 1485

Thr Phe Gly Gln Thr Met Tyr Thr Asp Ala Asp Glu Cys Val Leu Phe  
 5 1490 1495 1500

Gly Pro Ala Leu Cys Gln Asn Gly Arg Cys Ser Asn Ile Val Pro Gly  
 1505 1510 1515 1520

Tyr Ile Cys Leu Cys Asn Pro Gly Tyr His Tyr Asp Ala Ser Ser Arg  
 1525 1530 1535

Lys Cys Gln Asp His Asn Glu Cys Gln Asp Leu Ala Cys Glu Asn Gly  
 1540 1545 1550

Glu Cys Val Asn Gln Glu Gly Ser Phe His Cys Leu Cys Asn Pro Pro  
 1555 1560 1565

Leu Thr Leu Asp Leu Ser Gly Gln Arg Cys Val Asn Thr Thr Ser Ser  
 20 1570 1575 1580

Thr Glu Asp Phe Pro Asp His Asp Ile His Met Asp Ile Cys Trp Lys  
 1585 1590 1595 1600

-246-

Lys Val Thr Asn Asp Val Cys Ser Gln Pro Leu Arg Gly His His Thr  
1605 1610 1615

5 Thr Tyr Thr Glu Cys Cys Gln Asp Gly Glu Ala Trp Ser Gln Gln  
1620 1625 1630

Cys Ala Leu Cys Pro Pro Arg Ser Ser Glu Val Tyr Ala Gln Leu Cys  
1635 1640 1645

10 Asn Val Ala Arg Ile Glu Ala Glu Arg Gly Ala Gly Ile His Phe Arg  
1650 1655 1660

Pro Gly Tyr Glu Tyr Gly Pro Gly Leu Asp Asp Leu Pro Glu Asn Leu  
1665 1670 1675 1680

15 Tyr Gly Pro Asp Gly Ala Pro Phe Tyr Asn Tyr Leu Gly Pro Glu Asp  
1685 1690 1695

20 Thr Ala Pro Glu Pro Pro Phe Ser Asn Pro Ala Ser Gln Pro Gly Asp  
1700 1705 1710

Asn Thr Pro Val Leu Glu Glu Pro Pro Leu Gln Pro Ser Glu Leu Gln Pro  
1715 1720 1725

-247-

His Tyr Leu Ala Ser His Ser Glu Pro Pro Ala Ser Phe Glu Gly Leu  
1730 1735 1740

Gln Ala Glu Glu Cys Gly Ile Leu Asn Gly Cys Glu Asn Gly Arg Cys  
5 1745 1750 1755 1760

Val Arg Val Arg Glu Gly Tyr Thr Cys Asp Cys Phe Glu Gly Phe Gln  
1765 1770 1775

10 Leu Asp Ala Pro Thr Leu Ala Cys Val Asp Val Asn Glu Cys Glu Asp  
1780 1785 1790

Leu Asn Gly Pro Ala Arg Leu Cys Ala His Gly His Cys Glu Asn Thr  
1795 1800 1805

15 Glu Gly Ser Tyr Arg Cys His Cys Ser Pro Gly Tyr Val Ala Glu Pro  
1810 1815 1820

Gly Pro Pro His Cys Ala Ala Lys Glu \*  
20 1825 1830

- 248 -

CLAIMS

1. A method for transferring a nucleic acid segment  
5 into bone progenitor cells, comprising contacting bone progenitor cells with a composition comprising an isolated nucleic acid segment so as to transfer said nucleic acid segment into said cells.
- 10 2. The method of claim 1, wherein said cells are located within a bone progenitor tissue site of an animal and said tissue site is contacted with said composition so as to promote nucleic acid transfer into bone  
15 progenitor cells *in situ*.
3. The method of claim 2, wherein the contacting  
20 process comprises bringing said isolated nucleic acid segment into contact with a bone-compatible matrix to form a matrix-nucleic acid segment composition and bringing said matrix-nucleic acid segment composition into contact with said tissue site.
- 25 4. The use of a composition comprising an isolated nucleic acid segment and a bone-compatible matrix in the preparation of a formulation or medicament for transferring a nucleic acid segment into bone progenitor  
30 cells.
5. A use according to claim 4, wherein said formulation or medicament is intended for use in transferring a  
35 nucleic acid segment into bone progenitor cells within a bone progenitor tissue site of an animal.

- 249 -

6. A use according to claim 5, wherein said formulation or medicament is prepared by bringing an isolated nucleic acid segment into contact with a bone-compatible matrix to form a matrix-nucleic acid segment formulation or  
5 medicament intended for use in transferring a nucleic acid segment into bone progenitor cells within a bone progenitor tissue site of an animal.

10 7. A use according to claim 6, wherein said formulation or medicament is prepared by bringing an isolated nucleic acid segment into contact with a bone-compatible matrix and a pluronic agent to form a syringeable matrix-nucleic acid segment formulation or medicament.

15

8. A use according to claim 6, wherein said formulation or medicament further comprises a detectable agent for use in an imaging modality.

20

9. A use according to claim 8, wherein said formulation or medicament further comprises a radiographic agent.

25

10. A use according to claim 8, wherein said formulation or medicament further comprises a paramagnetic ion.

30 11. A use according to claim 8, wherein said formulation or medicament further comprises a radioactive ion.

12. A use according to claim 4, wherein said nucleic  
35 acid segment is a DNA molecule.

- 250 -

13. A use according to claim 4, wherein said nucleic acid segment is an RNA molecule.

5 14. A use according to claim 4, wherein said nucleic acid segment is an antisense nucleic acid molecule.

10 15. A use according to claim 4, wherein said nucleic acid segment is a linear nucleic acid molecule, a plasmid, a recombinant insert within the genome of a recombinant virus, or a nucleic acid segment associated with a liposome.

15 16. A use according to claim 15, wherein said nucleic acid segment is a nucleic acid segment associated with a liposome.

20 17. A use according to claim 4, wherein said nucleic acid segment encodes a polypeptide or protein that stimulates bone progenitor cells when expressed in said cells.

25 18. A use according to claim 6, wherein said bone-compatible matrix is a collagenous, metal, hydroxylapatite, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer matrix.

30

19. A use according to claim 18, wherein said bone-compatible matrix is a titanium matrix.

35



- 251 -

20. A use according to claim 19, wherein said bone-compatible matrix is a titanium matrix coated with hydroxylapatite.

5

21. A use according to claim 18, wherein said bone-compatible matrix is a collagen preparation.

10 22. A use according to claim 21, wherein said bone-compatible matrix is a type II collagen preparation.

15 23. A use according to claim 22, wherein said bone-compatible matrix is a type II collagen preparation obtained from hyaline cartilage.

20 24. A use according to claim 22, wherein said bone-compatible matrix is a recombinant type II collagen preparation.

25 25. A use according to claim 22, wherein said bone-compatible matrix is a mineralized type II collagen preparation.

30 26. A method of stimulating bone progenitor cells, comprising contacting bone progenitor cells with a composition comprising an isolated osteotropic gene so as to promote expression of said gene in said cells.

35 27. The method of claim 26, wherein said cells are located within a bone progenitor tissue site of an animal

- 252 -

and said tissue site is contacted with said composition  
so as to promote bone tissue growth.

- 5 28. The method of claim 27, wherein the contacting  
process comprises bringing said osteotropic gene into  
contact with a bone-compatible matrix to form a matrix-  
gene composition and bringing said matrix-gene  
composition into contact with said tissue site.
- 10
29. The use of a composition comprising an isolated  
osteotropic gene in the preparation of a formulation or  
medicament for use in promoting expression of the gene in  
15 bone progenitor cells and for stimulating said bone  
progenitor cells.
- 20 30. A use according to claim 29, wherein said  
formulation or medicament is intended for use in  
promoting expression of the gene in bone progenitor cells  
within a bone progenitor tissue site of an animal and for  
stimulating said bone progenitor cells to promote bone  
tissue growth.
- 25
31. A use according to claim 30, wherein said  
formulation or medicament is prepared by bringing an  
osteotropic gene into contact with a bone-compatible  
30 matrix to form a matrix-gene formulation or medicament  
intended for use in promoting expression of the gene in  
bone progenitor cells within a bone progenitor tissue  
site of an animal and for stimulating said bone  
progenitor cells to promote bone tissue growth.
- 35

- 253 -

32. A use according to claim 31, wherein said  
formulation or medicament is prepared by bringing an  
osteotropic gene into contact with a bone-compatible  
matrix and a pluronic agent to form a syringeable matrix-  
5 gene formulation or medicament.

33. A use according to claim 31, wherein said  
formulation or medicament further comprises a detectable  
10 agent for use in an imaging modality.

34. A use according to claim 33, wherein said  
formulation or medicament further comprises a  
15 radiographic agent.

35. A use according to claim 34, wherein said  
formulation or medicament further comprises calcium  
20 phosphate.

36. A use according to claim 33, wherein said  
formulation or medicament further comprises a  
paramagnetic ion.  
25

37. A use according to claim 36, wherein said  
formulation or medicament further comprises chromium  
(III), manganese (II), iron (III), iron (II), cobalt  
30 (II), nickel (II), copper (II), neodymium (III), samarium  
(III), ytterbium (III), gadolinium (III), vanadium (II),  
terbium (III), dysprosium (III), holmium (III) or  
erbium (III).

- 254 -

38. A use according to claim 33, wherein said formulation or medicament further comprises a radioactive ion.

5

39. A use according to claim 38, wherein said formulation or medicament further comprises iodine<sup>131</sup>, iodine<sup>123</sup>, technicium<sup>99m</sup>, indium<sup>111</sup>, rhenium<sup>188</sup>, rhenium<sup>186</sup>, gallium<sup>67</sup>, copper<sup>67</sup>, yttrium<sup>90</sup>, iodine<sup>125</sup> or astatine<sup>211</sup>.

10

40. A use according to claim 29, wherein said osteotropic gene is in the form of plasmid DNA, a DNA insert within the genome of a recombinant adenovirus, a DNA insert within the genome of a recombinant adeno-associated virus (AAV), a DNA insert within the genome of a recombinant retrovirus, or a DNA segment associated with a liposome.

15

20

41. A use according to claim 40, wherein said osteotropic gene is in the form of an osteotropic gene associated with a liposome.

25

42. A use according to claim 29, wherein said osteotropic gene is a parathyroid hormone (PTH) gene, a bone morphogenetic protein (BMP) gene, a growth factor gene, a growth factor receptor gene, a cytokine gene or a chemotactic factor gene.

30

43. A use according to claim 42, wherein said osteotropic gene is a transforming growth factor (TGF) gene, a fibroblast growth factor (FGF) gene, a granulocyte/macrophage colony stimulating factor (GMCSF)

35

- 255 -

gene, an epidermal growth factor (EGF) gene, a platelet derived growth factor (PDGF) gene, an insulin-like growth factor (IGF) gene, or a leukemia inhibitory factor (LIF) gene.

5

44. A use according to claim 43, wherein said osteotropic gene is a TGF- $\alpha$ , TGF- $\beta$ 1 or TGF- $\beta$ 2 gene.

10

45. A use according to claim 42, wherein said osteotropic gene is a PTH gene.

15

46. A use according to claim 42, wherein said osteotropic gene is a BMP gene.

20

47. A use according to claim 46, wherein said osteotropic gene is a BMP-2 or BMP-4 gene.

25

48. A use according to claim 31, wherein said bone-compatible matrix is a collagenous, metal, hydroxylapatite, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer matrix.

30

49. A use according to claim 48, wherein said bone-compatible matrix is a titanium matrix.

35

50. A use according to claim 49, wherein said bone-compatible matrix is a titanium matrix coated with hydroxylapatite.

- 256 -

51. A use according to claim 48, wherein said bone-compatible matrix is a collagen preparation.

5 52. A use according to claim 51, wherein said bone-compatible matrix is a type II collagen preparation.

53. A use according to claim 52, wherein said bone-compatible matrix is a type II collagen preparation  
10 obtained from hyaline cartilage.

54. A use according to claim 52, wherein said bone-compatible matrix is a recombinant type II collagen  
15 preparation.

55. A use according to claim 52, wherein said bone-compatible matrix is a mineralized type II collagen  
20 preparation.

56. A use according to claim 31, wherein said matrix-gene composition is applied to a bone fracture site in  
25 said animal.

57. A use according to claim 31, wherein said matrix-gene composition is implanted within a bone cavity site  
30 in said animal.

58. A use according to claim 31, wherein said bone cavity site is the result of dental or periodontal  
35 surgery or the removal of an osteosarcoma.

- 257 -

59. A composition comprising an isolated nucleic acid segment in association with a bone-compatible matrix.

5 60. The composition of claim 59, wherein said nucleic acid segment is a DNA molecule.

61. The composition of claim 59, wherein said nucleic  
10 acid segment is an RNA molecule.

62. The composition of claim 59, wherein said nucleic  
15 acid segment is an antisense nucleic acid molecule.

63. The composition of claim 59, wherein said nucleic  
acid segment is a linear nucleic acid molecule, a  
plasmid, a recombinant insert within the genome of a  
20 recombinant virus, or a nucleic acid segment associated  
with a liposome.

64. The composition of claim 63, wherein said nucleic  
acid segment is associated with a liposome.  
25

65. The composition of claim 59, wherein said nucleic  
acid segment encodes a polypeptide or protein that  
stimulates bone progenitor cells when expressed in said  
30 cells.

66. The composition of claim 59, wherein said bone-  
compatible matrix is a collagenous, titanium,  
35 hydroxylapatite, hydroxylapatite-coated titanium,  
bioglass, aluminate, bioceramic, acrylic ester polymer or

- 258 -

lactic acid polymer matrix.

67. The composition of claim 66, wherein said bone-  
5 compatible matrix is a collagen preparation.

68. The composition of claim 67, wherein said bone-  
compatible matrix is a type II collagen preparation.  
10

69. The composition of claim 68, wherein said bone-  
compatible matrix is a type II collagen preparation  
obtained from hyaline cartilage.  
15

70. The composition of claim 68, wherein said bone-  
compatible matrix is a recombinant type II collagen  
preparation.  
20

71. The composition of claim 68, wherein said bone-  
compatible matrix is a mineralized type II collagen  
preparation.  
25

72. The composition of claim 59, further defined as a  
syringeable composition.  
30

73. The composition of claim 59, wherein said  
composition further comprises a detectable agent for use  
in an imaging modality.  
35



- 259 -

74. The composition of claim 73, wherein said composition further comprises a radiographic agent.

5 75. The composition of claim 73, wherein said composition further comprises a paramagnetic ion.

76. The composition of claim 73, wherein said  
10 composition further comprises a radioactive ion.

77. A composition comprising an isolated osteotropic gene in association with a bone-compatible matrix, said composition being capable of stimulating bone growth when  
15 administered to a bone progenitor tissue site of an animal.

78. The composition of claim 77, wherein said  
20 osteotropic gene is in the form of plasmid DNA, a DNA insert within the genome of a recombinant adenovirus, a DNA insert within the genome of a recombinant adeno-associated virus (AAV), a DNA insert within the genome of a recombinant retrovirus, or a DNA segment associated  
25 with a liposome.

79. The composition of claim 78, wherein said osteotropic gene is in the form of an osteotropic gene  
30 associated with a liposome.

80. The composition of claim 77, wherein said osteotropic gene is a PTH, BMP, TGF- $\alpha$ , TGF- $\beta$ 1, TGF- $\beta$ 2, FGF, GMCSF, EGF, PDGF, IGF or a LIF gene.  
35

- 260 -

81. The composition claim 80, wherein said osteotropic gene is a TGF- $\alpha$ , TGF- $\beta$ 1, TGF- $\beta$ 2, PTH, BMP-2 or BMP-4 gene.

5

82. The composition of claim 77, wherein said bone-compatible matrix is a collagenous, metal, hydroxyapatite, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer matrix.

10

83. The composition of claim 82, wherein said bone-compatible matrix is a titanium matrix.

15

84. The composition of claim 83, wherein said bone-compatible matrix is a titanium matrix coated with hydroxylapatite.

20

85. The composition of claim 82, wherein said bone-compatible matrix is a collagen preparation.

25

86. The composition of claim 85, wherein said bone-compatible matrix is a type II collagen preparation.

30

87. The composition of claim 86, wherein said bone-compatible matrix is a type II collagen preparation obtained from hyaline cartilage.

35

88. The composition of claim 86, wherein said bone-compatible matrix is a recombinant type II collagen preparation.

- 261 -

89. The composition of claim 86, wherein said bone-compatible matrix is a mineralized type II collagen preparation.

5

90. The composition of claim 77, further defined as comprising an isolated osteotropic gene in association with a bone-compatible matrix and a pluronic agent, the composition forming a syringeable composition.

10

91. The composition of claim 77, wherein said composition further comprises a detectable agent for use in an imaging modality.

15

92. The composition of claim 91, wherein said composition further comprises a radiographic agent.

20

93. The composition of claim 92, wherein said composition further comprises calcium phosphate.

25

94. The composition of claim 91, wherein said composition further comprises a paramagnetic ion.

30

95. The composition of claim 94, wherein said composition further comprises chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) or erbium (III).

35

- 262 -

96. The composition of claim 91, wherein said composition further comprises a radioactive ion.

5 97. The composition of claim 96, wherein said composition further comprises iodine<sup>131</sup>, iodine<sup>123</sup>, technetium<sup>99m</sup>, indium<sup>111</sup>, rhenium<sup>188</sup>, rhenium<sup>186</sup>, gallium<sup>67</sup>, copper<sup>67</sup>, yttrium<sup>90</sup>, iodine<sup>125</sup> or astatine<sup>211</sup>.

10

98. A kit comprising, in suitable container means, a pharmaceutically acceptable bone-compatible matrix and a pharmaceutically acceptable osteotropic gene preparation.

15

99. The kit of claim 98, wherein said bone-compatible matrix is a collagenous, titanium, hydroxylapatite, hydroxylapatite-coated titanium, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer  
20 matrix.

100. The kit of claim 99, wherein said bone-compatible matrix is a titanium matrix.

25

101. The kit of claim 99, wherein said bone-compatible matrix is a hydroxylapatite-coated titanium matrix.

30 102. The kit of claim 99, wherein said bone-compatible matrix is a collagenous matrix.

103. The kit of claim 102, wherein said bone-compatible  
35 matrix is a type II collagen matrix.

- 263 -

104. The kit of claim 103, wherein said bone-compatible matrix is a type II collagen matrix obtained from hyaline cartilage.

5

105. The kit of claim 103, wherein said bone-compatible matrix is a recombinant type II collagen matrix.

10 106. The kit of claim 103, wherein said bone-compatible matrix is a mineralized type II collagen matrix.

15 107. The kit of claim 98, wherein said osteotropic gene preparation comprises a linear osteotropic gene, a plasmid including an osteotropic gene, a recombinant virus having a genome that includes an osteotropic gene or an osteotropic gene associated with a liposome.

20

108. The kit of claim 98, wherein said osteotropic gene preparation comprises a lyophilized gene preparation.

25 109. The kit of claim 98, wherein said osteotropic gene preparation comprises a PTH, TGF, BMP, FGF, GMCSF, EGF, PDGF, IGF or a LIF gene.

30 110. The kit of claim 109, wherein said osteotropic gene preparation comprises a PTH, TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3, BMP-2 or a BMP-4 gene.

35 111. The kit of claim 98, further comprising a pluronic agent.

- 264 -

112. The kit of claim 98, further comprising a detectable agent for use in an imaging modality.

5

113. The kit of claim 112, wherein said composition further comprises a radiographic agent.

10 114. The kit of claim 113, wherein said composition further comprises calcium phosphate.

115. The kit of claim 112, wherein said composition  
15 further comprises a paramagnetic ion.

116. The kit of claim 115, wherein said composition  
further comprises chromium (III), manganese (II), iron  
20 (III), iron (II), cobalt (II), nickel (II), copper (II),  
neodymium (III), samarium (III), ytterbium (III),  
gadolinium (III), vanadium (II), terbium (III),  
dysprosium (III), holmium (III) or erbium (III).

25

117. The kit of claim 112, wherein said composition  
further comprises a radioactive ion.

30 118. The kit of claim 117, wherein said composition  
further comprises iodine<sup>131</sup>, iodine<sup>123</sup>, technicium<sup>99m</sup>,  
indium<sup>111</sup>, rhenium<sup>188</sup>, rhenium<sup>186</sup>, gallium<sup>67</sup>, copper<sup>67</sup>,  
yttrium<sup>90</sup>, iodine<sup>125</sup> or astatine<sup>211</sup>.

35

119. The kit of claim 98, wherein said bone-compatible

- 265 -

matrix and said osteotropic gene preparation are present within a single container means.

5    120. The kit of claim 119, wherein said container means is a syringe or pipette.

10    121. The kit of claim 98, wherein said bone-compatible matrix and said osteotropic gene preparation are present within distinct container means.

15    122. The kit of claim 98, further comprising a third container means comprising a pharmaceutically acceptable diluent.

20    123. The kit of claim 98, further comprising a syringe, pipette or forceps.

25    124. An osteotropic device, comprising an isolated osteotropic gene capable of expression in bone progenitor cells, the gene associated with an amount of a bone-compatible matrix effective to absorb said gene, wherein said device is capable of stimulating bone formation when implanted within a bone progenitor tissue site of an animal.

30    125. The device of claim 124, wherein said device is a titanium or a hydroxylapatite-coated titanium device.

35    126. The device of claim 124, wherein said device is shaped to join a bone fracture site in said animal.

- 266 -

127. The device of claim 124, wherein said device is shaped to fill a bone cavity site in said animal.

5

128. The device of claim 124, wherein said device is an artificial joint.

10 129. A DNA segment comprising an isolated gene that encodes a protein or peptide that includes an amino acid sequence essentially as set forth by a contiguous sequence from SEQ ID NO:3.

15

130. The DNA segment of claim 129, comprising an isolated gene that includes a nucleic acid sequence essentially as set forth by a contiguous sequence from SEQ ID NO:2.

20

131. A composition comprising a purified protein that has an amino acid sequence essentially as set forth by the amino acid sequence of SEQ ID NO:3.

25

132. A method for stimulating a bone progenitor cell, comprising contacting a bone progenitor cell with a composition comprising a biologically effective amount of type II collagen.

30

133. The use of a composition comprising a biologically effective amount of type II collagen in the preparation of a formulation or medicament for stimulating a bone progenitor cell.

35



- 267 -

134. A use according to claim 133, wherein said composition comprises type II collagen purified from hyaline cartilage.

5

135. A use according to claim 133, wherein said composition comprises recombinant type II collagen.

10

136. A use according to claim 133, wherein said composition comprises type II collagen further supplemented with minerals.

15

137. A use according to claim 136, wherein said composition comprises type II collagen further supplemented with calcium.

20

138. A use according to claim 133, wherein said composition comprises between about 1 mg and about 500 mg of type II collagen.

25

139. A use according to claim 138, wherein said composition comprises between about 1 mg and about 100 mg of type II collagen.

30

140. A use according to claim 139, wherein said composition comprises about 10 mg of type II collagen.

35

141. A use according to claim 133, wherein said composition comprises type II collagen in combination with a nucleic acid segment that encodes a polypeptide or

- 268 -

protein that stimulates bone progenitor cells when expressed in said cells.

5     142. A use according to claim 141, wherein said nucleic acid segment comprises an isolated PTH gene, BMP gene, growth factor gene, growth factor receptor gene, cytokine gene or chemotactic factor gene.

10

143. A use according to claim 142, wherein said nucleic acid segment comprises an isolated BMP gene.

15     144. A use according to claim 143, wherein said nucleic acid segment comprises an isolated BMP-2 or BMP-4 gene.

20     145. A use according to claim 141, wherein said composition further comprises a detectable agent for use in an imaging modality.

25     146. A use according to claim 133, wherein said formulation or medicament is intended for use in stimulating a bone progenitor cell located within a bone progenitor tissue site of an animal and for promoting bone tissue growth.

30

147. A use according to claim 146, wherein said formulation or medicament is intended for use in implantation within a bone cavity site in an animal and for promoting bone tissue growth in said bone cavity  
35     site.

- 269 -

148. A use according to claim 146, wherein said  
formulation or medicament is intended for use in  
implantation within a bone fracture site in an animal and  
for promoting bone tissue growth in said bone fracture  
5 site.

149. A method for promoting bone growth, comprising  
contacting a bone progenitor tissue site of an animal  
10 with a composition comprising type II collagen in an  
amount effective to activate bone progenitor cells of  
said tissue site.

15 150. The use of a composition comprising a biologically  
effective amount of type II collagen in the preparation  
of a formulation or medicament for promoting bone growth  
in a bone progenitor tissue site of an animal.

20 151. A use according to claim 150, wherein said  
composition comprises recombinant type II collagen.

25 152. A use according to claim 150, wherein said  
composition comprises type II collagen further  
supplemented with minerals.

30 153. A use according to claim 150, wherein said  
composition comprises type II collagen and an osteotropic  
gene in a combined amount effective to activate bone  
progenitor cells of said tissue site.

35

- 270 -

154. A use according to claim 153, wherein said composition comprises type II collagen in combination with a PTH, TGF- $\beta$  or BMP gene.

5

155. A use according to claim 153, wherein said composition further comprises a detectable agent for use in an imaging modality.

1/45

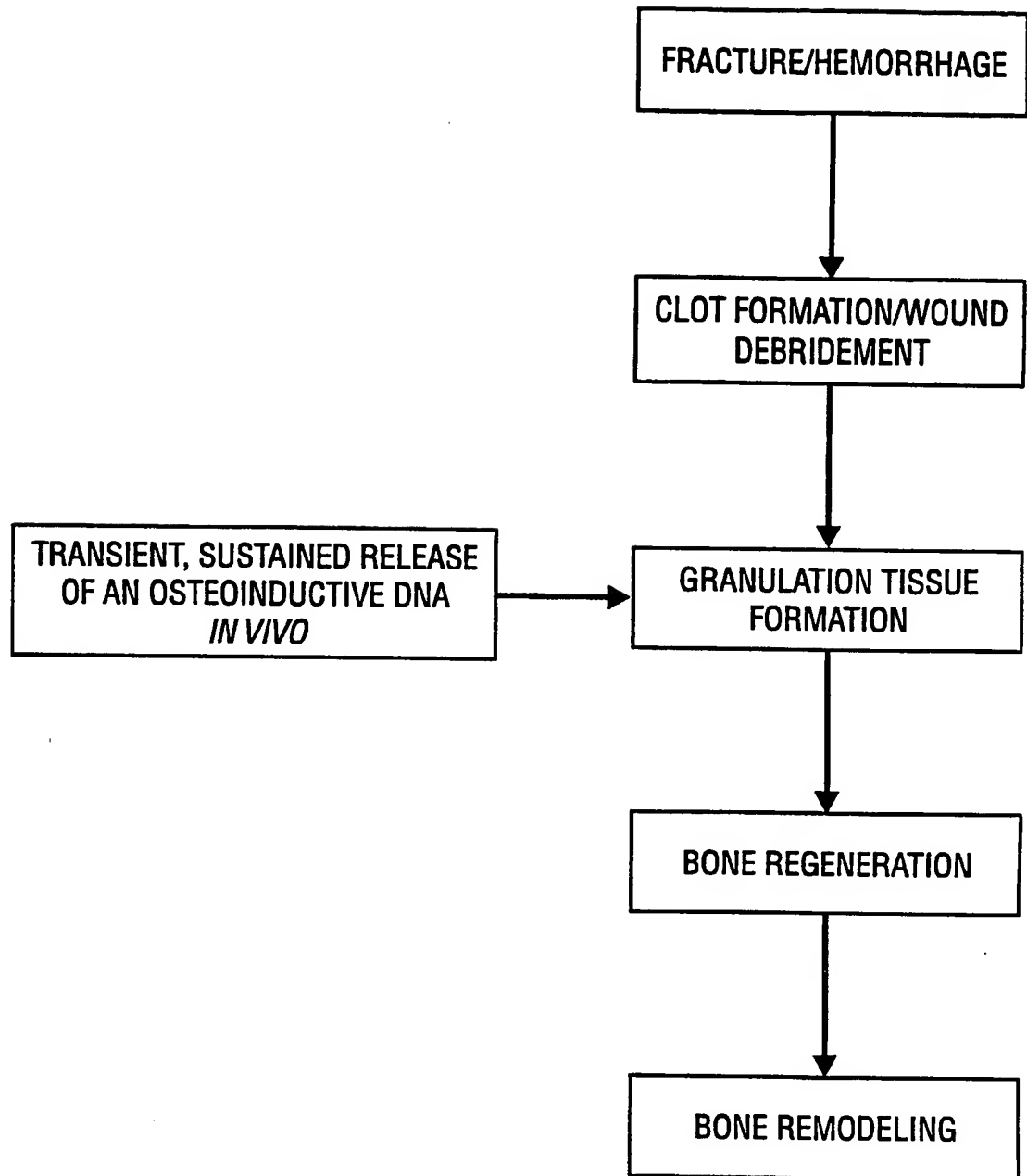


FIG. 1

2/45

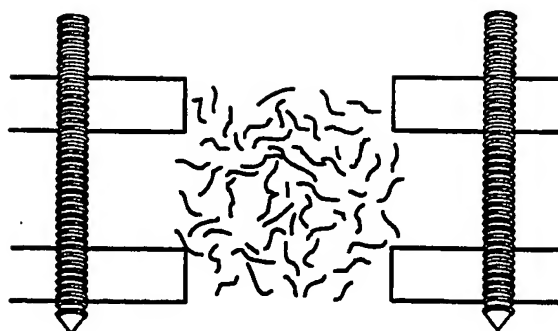


FIG. 2A

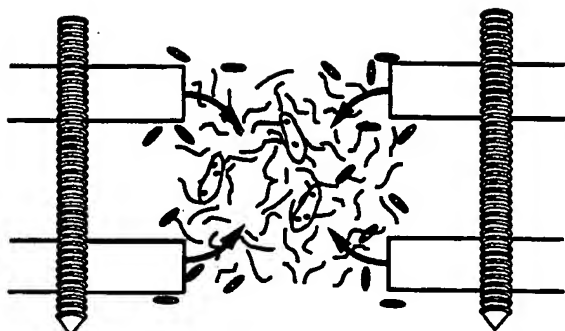


FIG. 2B

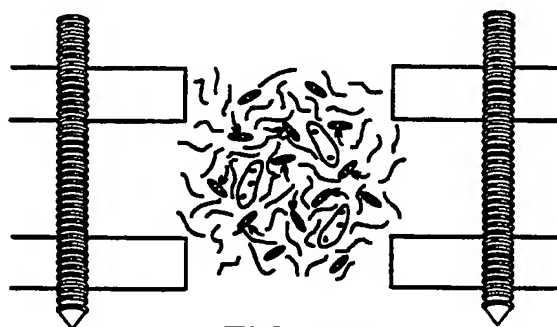


FIG. 2C

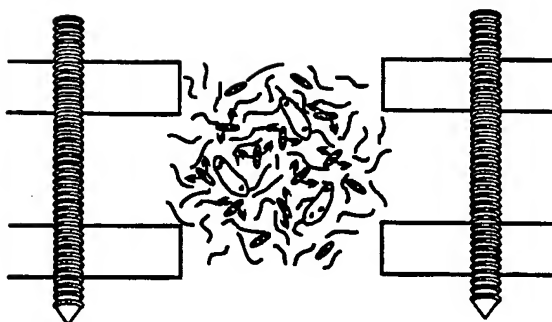


FIG. 2D

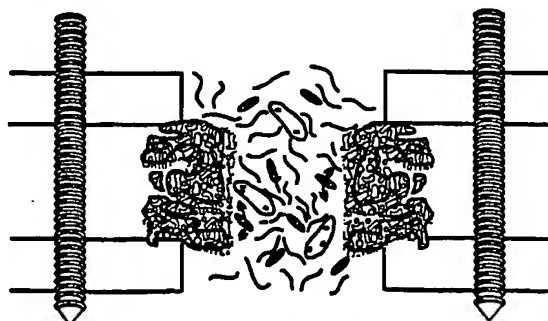


FIG. 2E

3/45

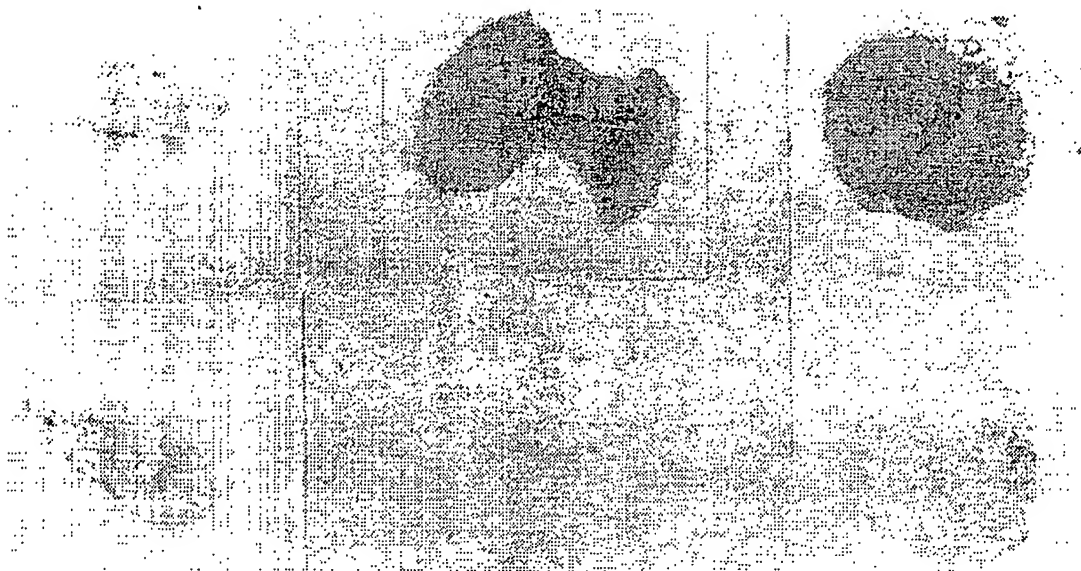


FIG. 3A

FIG. 3B

FIG. 3C

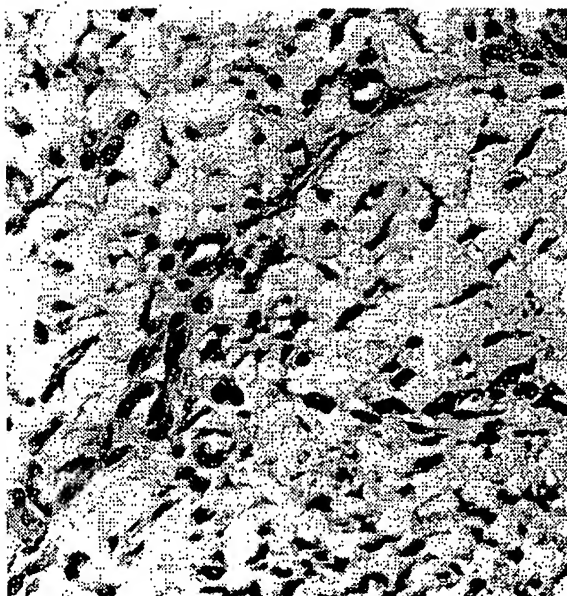


FIG.3D



FIG. 3E

4/45

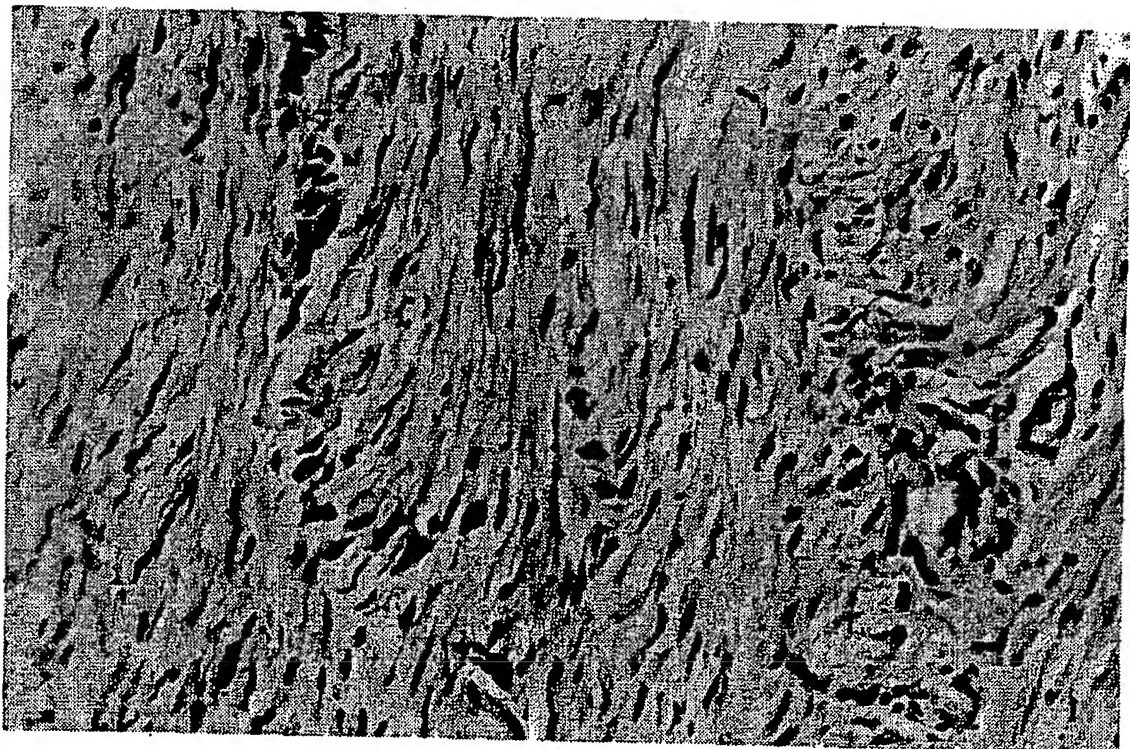


FIG. 4

SUBSTITUTE SHEET (RULE 26)



5/45

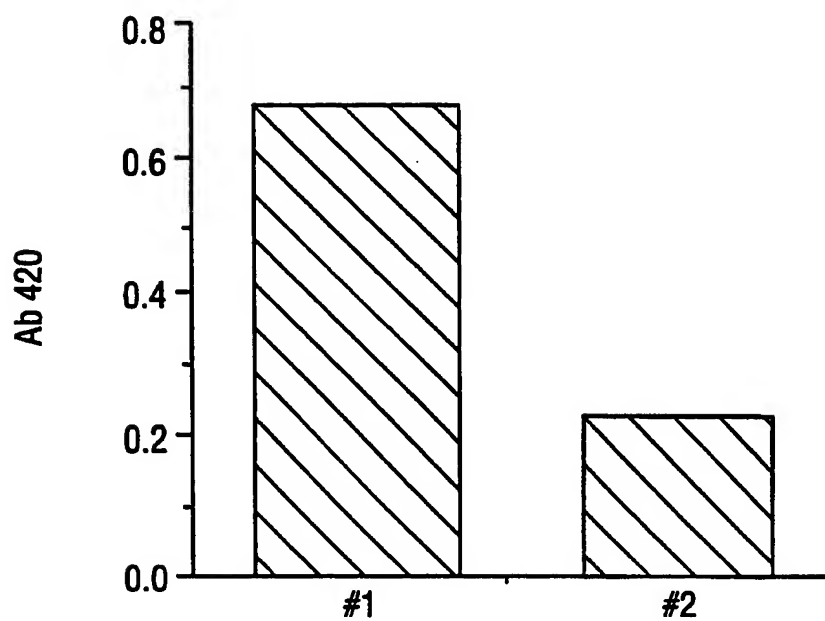


FIG. 5A

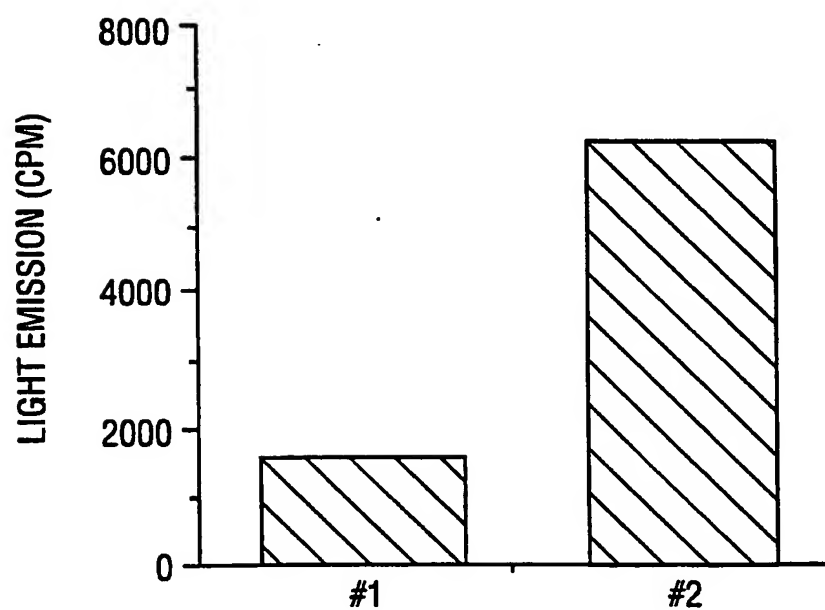


FIG. 5B

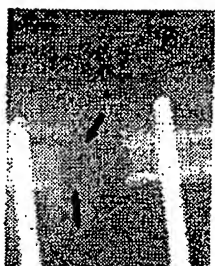


FIG. 6A

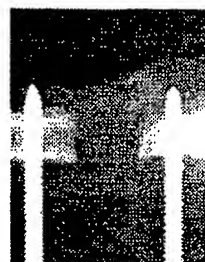


FIG. 6B

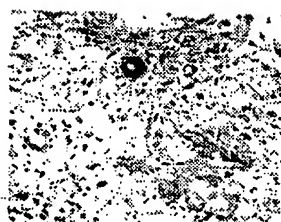


FIG. 6C

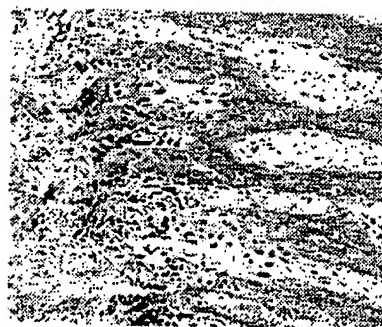


FIG. 6D

7/45

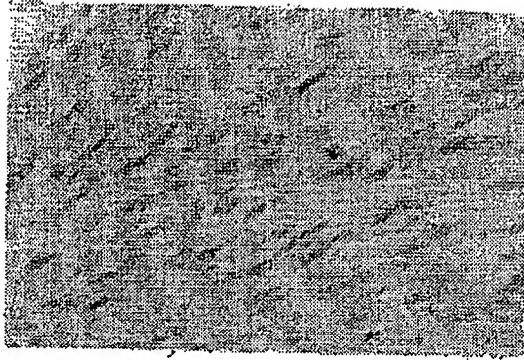


FIG. 7A

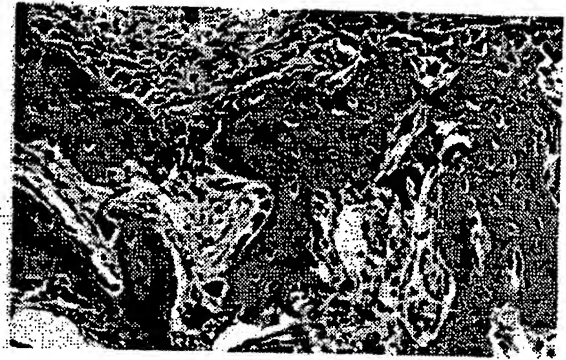


FIG. 7B

8/45

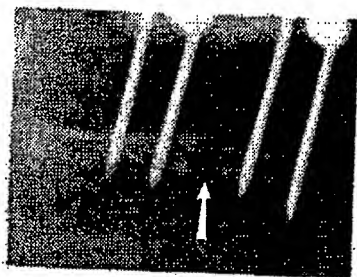


FIG. 8A

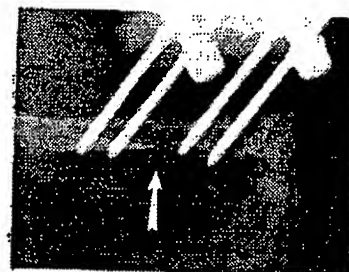


FIG. 8B



FIG. 8C

9/45

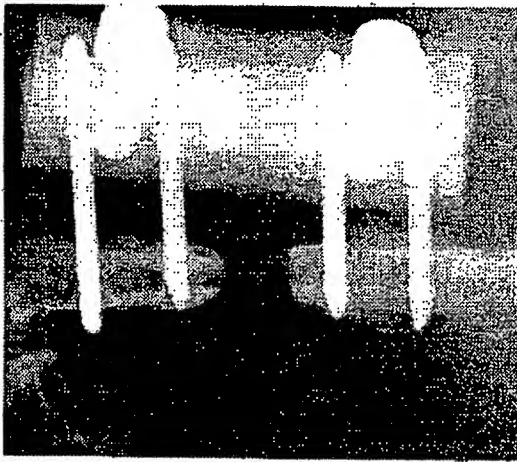


FIG. 9A

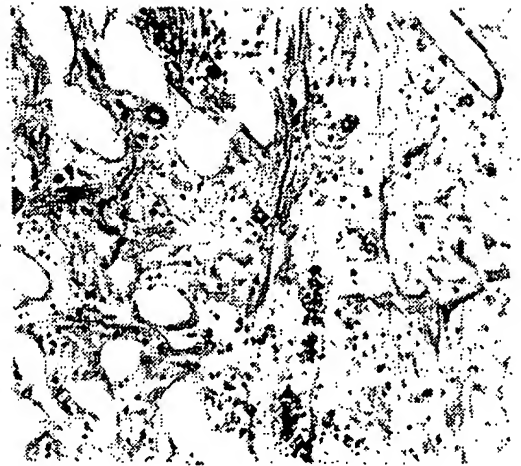


FIG. 9B

10/45

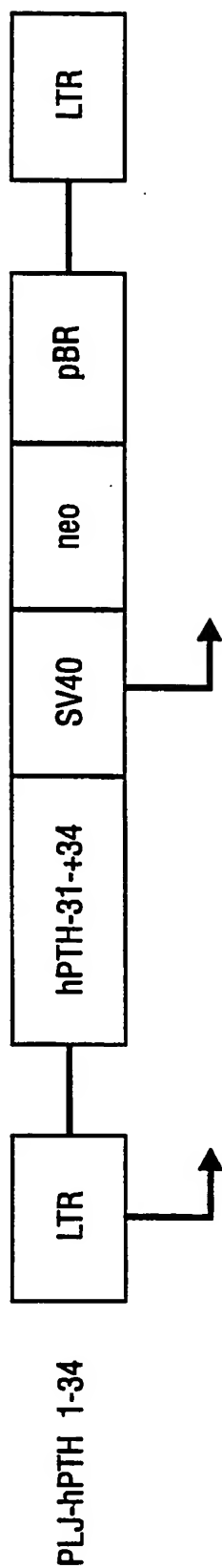


FIG. 10

11/45

1 2 3 4

4.3—

FIG. 11

12/45

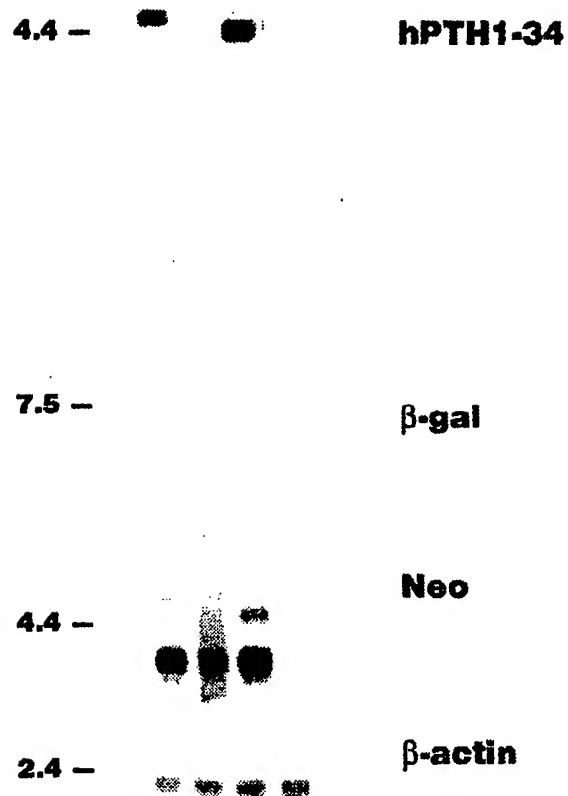


FIG. 12



13/45

CONTROL  
FEMUR

OSTEOTOMY  
FEMUR

FIG. 13

14/45

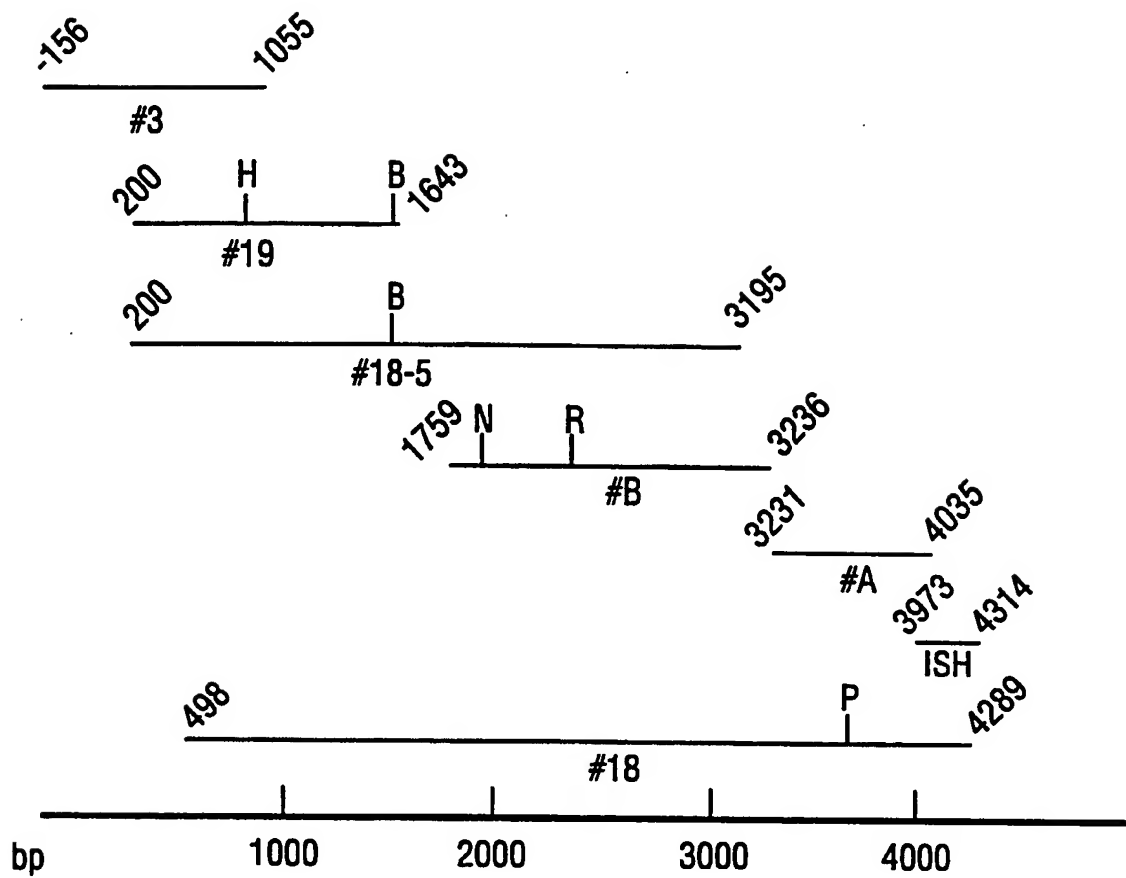
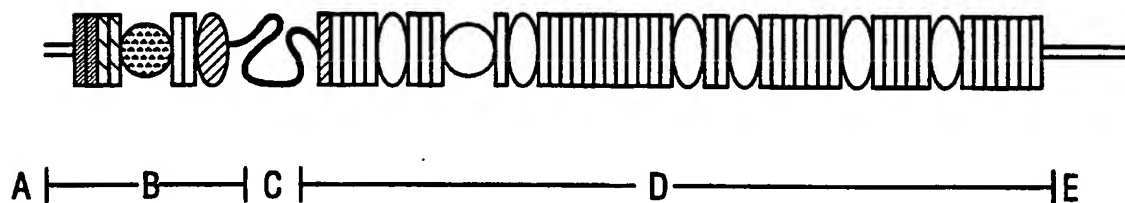


FIG. 14

15/45



**FIG. 15A**

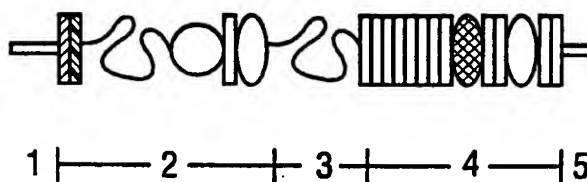


FIG. 15B

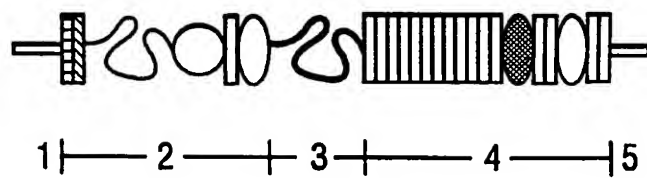


FIG. 15C

16/45

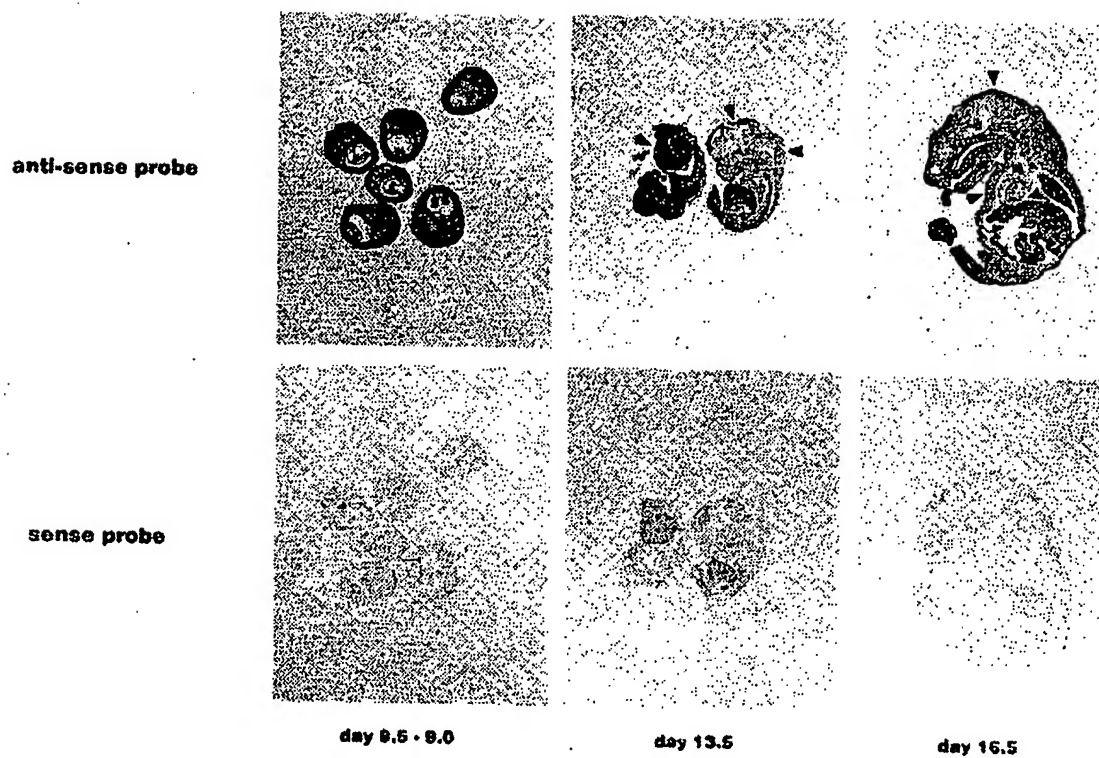


FIG. 16

17/45



FIG. 17A

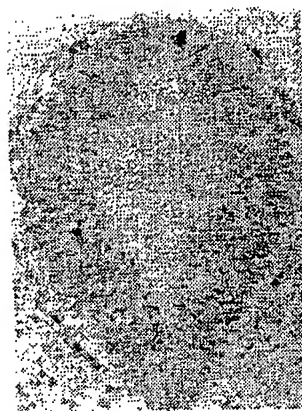


FIG. 17B

18/45

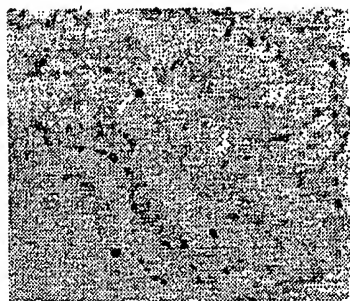


FIG. 17C



FIG. 17D

19/45

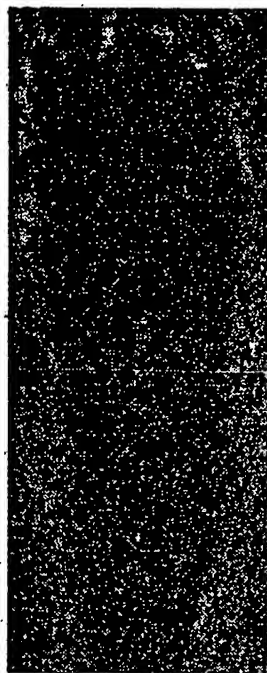


FIG. 18A

20/45

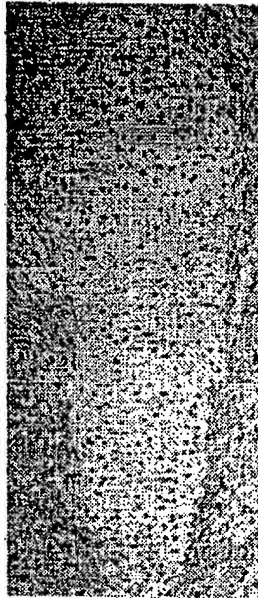


FIG. 18B



21/45

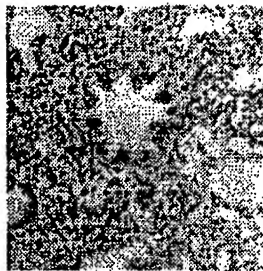


FIG. 18C

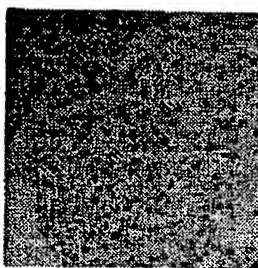


FIG. 18D

22/45

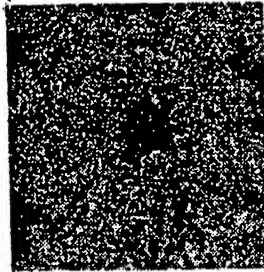


FIG. 18E

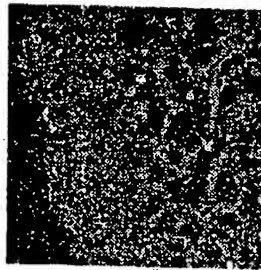


FIG. 18F

23/45



FIG. 18G



FIG. 18H

24/45

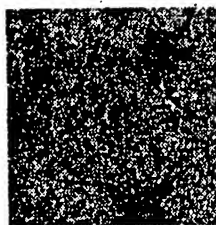


FIG. 18I

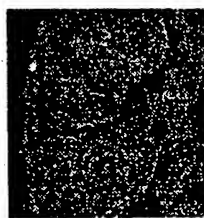


FIG. 18J

25/45



FIG. 18K



FIG. 18L

26/45

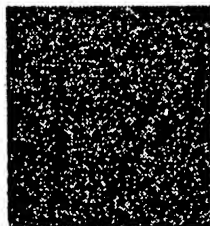


FIG. 18M



FIG. 18N

27/45



FIG. 180

28/45

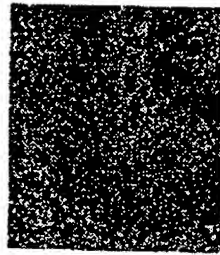
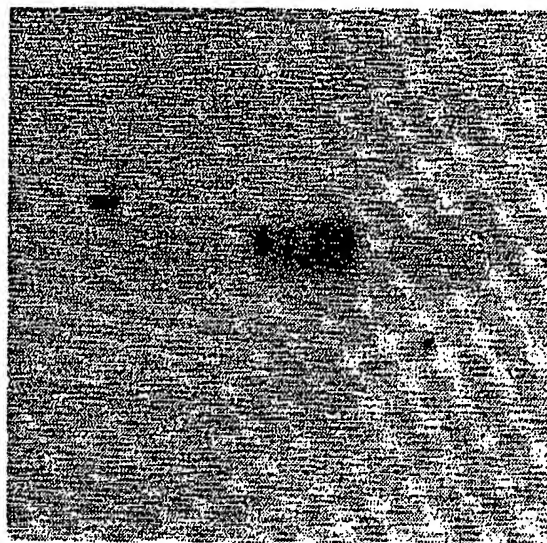


FIG. 18P



29/45

4.4 kb—



**DAY**

**5**

**14**

**28**

**FIG. 19**

30/45

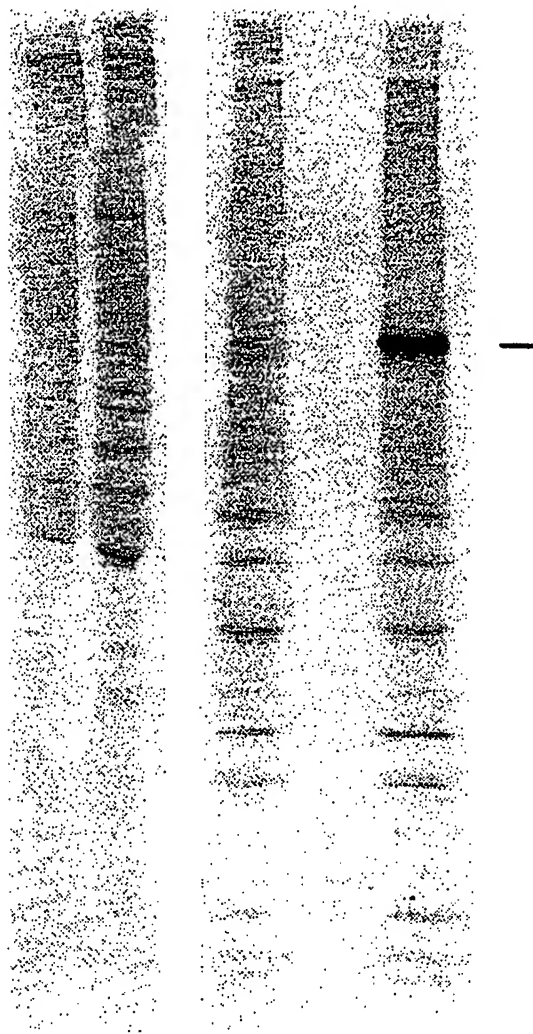


FIG. 20

31/45

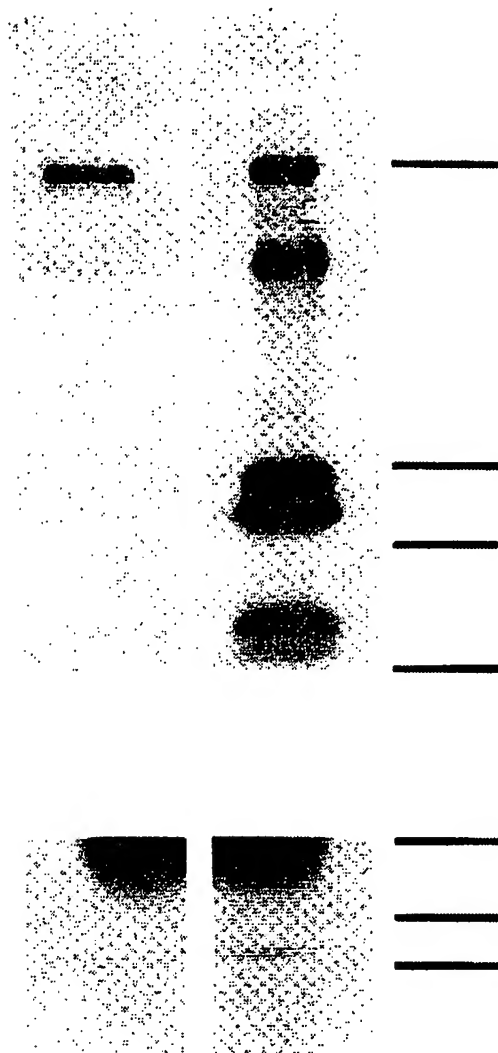


FIG. 21

32/45

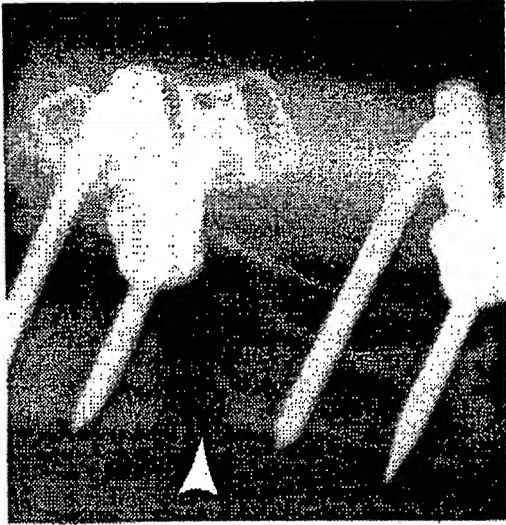


FIG. 22A



FIG. 22B

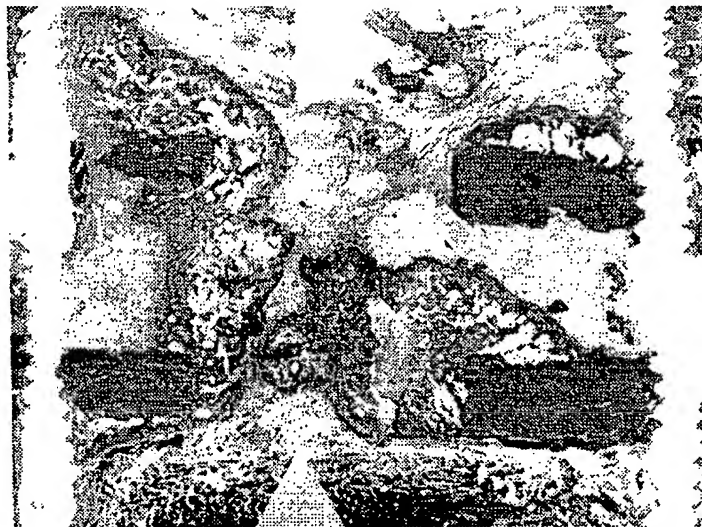


FIG. 22C

33/45

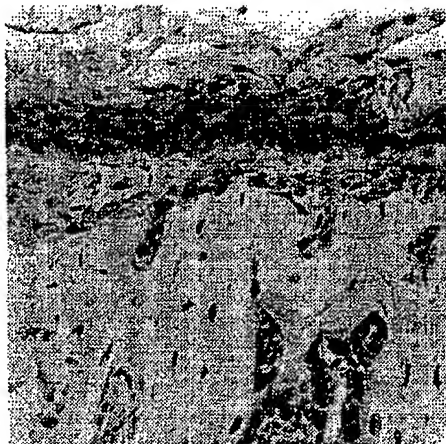


FIG. 23A



FIG. 23B



FIG. 23C

34/45

MIPGNRMLNV VLLCQVLLGG ATDASLMPET GKKKVAEIQG HAGRRSGQS HELLRDFEAT LLQMFGLRRR  
PQPSKSAVIP DYMSDLYRLQ SGEEEEEEQS QGTGLEYPER PASSANTVSS FHHEHLENI PGTSSESAFR  
FFFNLSIPE NEVISSAELR LFREQVDQGP DWEOGFHRMN IYEVMKPPAE MYPGHLITRL LDTSLVRHNV  
TRWETFDVSP AVLRWTREKQ PNYGLAIEVT HLHQTRTHQG QHVSISRSLP QSGNWAQLR PLLVTFGHDG  
RGHTLTRRSA KRSPKHPQR SSKKNKNCRR HSLYVDFSDV GWNDWIVAPP GYQAFYCHGD CPFPLADHLN  
STNHAIVOTL VNSVNSSIPK ACCVPTLSA ISMLYLDEYD KVLKNYQEM VVEGCGCRYP YDVPDYA

FIG. 24

35/45

ATG CGC CAG GCC GCA TTG GGG CTG CTG GCA CTA CTC CTG CTG GCG CTG CTG GGC 54  
 M R Q A A L G L L A L L L L A L L G 18

CCC GGC GGC CGA GGG GTG GGC CGG CCG GGC AGC GGG GCA CAG GCG GGG GCG GGG 108  
 P G G R G V G R P G S G A Q A G A G 36

CGC TGG GCC CAA CGC TTC AAG GTG GTC TTT GCG CCT GTG ATC TGC AAG CGG ACC 162  
 R W A Q R F K V V F A P V I C K R T 54

TGT CTG AAG GGC CAG TGT CGG GAC AGC TGT CAG CAG GGC TCC AAC ATG ACG CTC 216  
 C L K G Q C R D S C Q Q G S N M T L 72

ATC GGA GAG AAC GGC CAC AGC ACC GAC ACG CTC ACC GGT TCT GCC TTC CGC GTG 270  
 I G E N G H S T D T L T G S A F R V 90

GTG GTG TGC CCT CTA CCC TGC ATG AAC GGT GGC CAG TGC TCT TCC CGA AAC CAG 324  
 V V C P L P C M N G G Q C S S R N Q 108

TGC CTG TGT CCC CCG GAT TTC ACG GGG CGC TTC TGC CAG GTG CCT GCT GCA GGA 378  
 C L C P P D F T G R F C Q V P A A G 126

ACC GGA GCT GGC ACC GGG AGT TCA GGC CCC GGC TGG CCC GAC CGG GCC ATG TCC 432  
 T G A G T G S S G P G W P D R A M S 144

ACA GGC CCG CTG CCG CCC CTT GCC CCA GAA GGA GAG TCT GTG GCT AGC AAA CAC 486  
 T G P L P P L A P E G E S V A S K H 162

GCC ATT TAC GCG GTG CAG GTG ATC GCA GAT CCT CCC GGG CCG GGG GAG GGT CCT 540  
 A I Y A V Q V I A D P P G P G E G P 180

CCT GCA CAA CAT GCA GCC TTC TTG GTG CCC CTG GGG CCA GGA CAA ATC TCG GCA 594  
 P A Q H A A F L V P L G P G Q I S A 198

GAA GTG CAG GCT CCG CCC CCC GTG GTG AAC GTG CGT GTC CAT CAC CCT CCT GAA 648  
 E V Q A P P P V V N V R V H H P P E 216

GCT TCC GTT CAG GTG CAC CGC ATC GAG GGG CCG AAC GCT GAA GGC CCA GCC TCT 702  
 A S V Q V H R I E G P N A E G P A S 234

TCC CAG CAC TTG CTG CCG CAT CCC AAG CCC CAG CAC CCG AGG CCA CCC ACT CAA 756  
 S Q H L L P H P K P Q H P R P P T Q 252

AAG CCA CTG GGC CGC TGC TTC CAG GAC ACA TTG CCC AAG CAG CCT TGT GGC AGC 810  
 K P L G R C F Q D T L P K Q P C G S 270

AAC CCT TTG CCT GGC CTT ACC AAG CAG GAA GAT TGC TGC GGT AGC ATC GGT ACT 864  
 N P L P G L T K Q E D C C G S I G T 288

GCC TGG GGA CAA AGC AAG TGT CAC AAG TGC CCA CAG CTT CAG TAT ACA GGG GTG 918  
 A W G Q S K C H K C P Q L Q Y T G V 306

CAG AAG CCT GTA CCT GTA CGT GGG GAG GTG GGT GCT GAC TGC CCC CAG GGC TAC 972  
 Q K P V P V R G E V G A D C P Q G Y 324

AAG AGG CTC AAC AGC ACC CAC TGC CAG GAT ATC AAC GAA TGT GCG ATG CCC GGG 1026  
 K R L N S T H C Q D I N E C A M P G 342

FIG. 25-1

SUBSTITUTE SHEET (RULE 26)

36/45

AAT	GTG	TGC	CAT	GGT	GAC	TGC	CTC	AAC	AAC	CCT	GGC	TCT	TAT	CGC	TGT	GTC	TGC	1080
N	V	C	H	G	D	C	L	N	N	P	G	S	Y	R	C	V	C	360
CCG	CCC	GGT	CAT	AGC	TTG	GGT	CCC	CTC	GCA	GCA	CAG	TGC	ATT	GCC	GAC	AAA	CCA	1134
P	P	G	H	S	L	G	P	L	A	A	Q	C	I	A	D	K	P	378
GAG	GAG	AAG	AGC	CTG	TGT	TTC	CGC	CTT	GTG	AGC	ACC	GAA	CAC	CAG	TGC	CAG	CAC	1188
E	E	K	S	L	C	F	R	L	V	S	T	E	H	Q	C	Q	H	396
CCT	CTG	ACC	ACA	CGC	CTA	ACC	CGC	CAG	CTC	TGC	TGC	TGT	AGT	GTG	GGT	AAA	GCC	1242
P	L	T	T	R	L	T	R	Q	L	C	C	C	S	V	G	K	A	414
TGG	GGT	GCC	CGG	TGC	CAG	CGC	TGC	CCG	GCA	GAT	GGT	ACA	GCA	GCC	TTC	AAG	GAG	1296
W	G	A	R	C	Q	R	C	P	A	D	G	T	A	A	F	K	E	432
ATC	TGC	CCC	GGC	TGG	GAA	AGG	GTA	CCA	TAT	CCT	CAC	CTC	CCA	CCA	GAC	GCT	CAC	1350
I	C	P	G	W	E	R	V	P	Y	P	H	L	P	P	D	A	H	450
CAT	CCA	GGG	GGA	AAG	CGA	CTT	CTC	CCT	CTT	CCT	GCA	CCC	GAC	GGG	CCA	CCC	AAA	1404
H	P	G	G	K	R	L	L	P	L	P	A	P	D	G	P	P	K	468
CCC	CAG	CAG	CTT	CCT	GAA	AGC	CCC	AGC	CGA	GCA	CCA	CCC	CTC	GAG	GAC	ACA	GAG	1458
P	Q	Q	L	P	E	S	P	S	R	A	P	P	L	E	D	T	E	486
GAA	GAG	AGA	GGA	GTG	ACC	ATG	GAT	CCA	CCA	GTG	AGT	GAG	GAG	CGA	TCG	GTG	CAG	1512
E	E	R	G	V	T	M	D	P	P	V	S	E	E	R	S	V	Q	504
CAG	AGC	CAC	CCC	ACT	ACC	ACC	ACC	TCA	CCC	CCC	CGG	CCT	TAC	CCA	GAG	CTC	ATC	1566
Q	S	H	P	T	T	T	T	S	P	P	R	P	Y	P	E	L	I	522
TCT	CGC	CCC	TCC	CCA	CCT	ACC	TTC	CAC	CGG	TTC	CTG	CCA	GAC	TTG	CCC	CCA	TCC	1620
S	R	P	S	P	P	T	F	H	R	F	L	P	D	L	P	P	S	540
CGA	AGT	GCA	GTG	GAG	ATC	GCC	CCC	ACT	CAG	GTC	ACA	GAG	ACC	GAT	GAG	TGC	CGA	1674
R	S	A	V	E	I	A	P	T	Q	V	T	E	T	D	E	C	R	558
TTG	AAC	CAG	AAT	ATC	TGT	GGC	CAT	GGA	CAG	TGT	GTG	CCT	GGC	CCC	TCG	GAT	TAC	1728
L	N	Q	N	I	C	G	H	G	Q	C	V	P	G	P	S	D	Y	576
TCC	TGC	CAC	TGC	AAC	GCT	GGC	TAC	CGG	TCA	CAC	CCG	CAG	CAC	CGC	TAC	TGT	GTT	1782
S	C	H	C	N	A	G	Y	R	S	H	P	Q	H	R	Y	C	V	594
GAT	GTG	AAC	GAG	TGC	GAG	GCA	GAG	CCC	TGC	GGC	CCC	GGG	AAA	GGC	ATC	TGT	ATG	1836
D	V	N	E	C	E	A	E	P	C	G	P	G	K	G	I	C	M	612
AAC	ACT	GGT	GGC	TCC	TAC	AAT	TGT	CAC	TGC	AAC	CGA	GGC	TAC	CGC	CTC	CAC	GTG	1890
N	T	G	G	S	Y	N	C	H	C	N	R	G	Y	R	L	H	V	630
GGT	GCA	GGG	GGC	CGC	TCG	TGC	GTG	GAC	CTG	AAC	GAG	TGC	GCC	AAG	CCT	CAC	CTG	1944
G	A	G	G	R	S	C	V	D	L	N	E	C	A	K	P	H	L	648
TGT	GGG	GAC	GGT	GGC	TTC	TGC	ATC	AAC	TTC	CCT	GGT	CAC	TAC	AAA	TGC	AAC	TGC	1998
C	G	D	G	G	F	C	I	N	F	P	G	H	Y	K	C	N	C	666
TAT	CCT	GGC	TAC	CGG	CTC	AAG	GCC	TCC	CGA	CCG	CCC	ATT	TGC	GAA	GAC	ATC	GAC	2052
Y	P	G	Y	R	L	K	A	S	R	P	P	I	C	E	D	I	D	684
GAG	TGT	CGC	GAC	CCT	AGC	ACC	TGC	CCT	GAT	GGC	AAA	TGT	GAA	AAC	AAA	CCT	GGC	2106
E	C	R	D	P	S	T	C	P	D	G	K	C	E	N	K	P	G	702

FIG. 25-2

SUBSTITUTE SHEET (RULE 26)



37/45

AGC TTC AAG TGC ATC GCC TGC CAG CCT GGC TAC CGT AGC CAG GGG GGC GGG GCC 2160  
 S F K C I A C Q P G Y R S Q G G G A 720  
 TGT CGT GAT GTC AAC GAA TGC TCC GAA GGT ACC CCC TGC TCT CCT GGA TGG TGT 2214  
 C R D V N E C S E G T P C S P G W C 738  
 GAG AAA CTT CCG GGT TCT TAC CGT TGC ACG TGT GCC CAG GGG ATA CGA ACC CGC 2268  
 E K L P G S Y R C T C A Q G I R T R 756  
 ACA GGA CGC CTC AGT TGC ATA GAC GTG GAT GAC TGT GAG GCT GGG AAA GTG TGC 2322  
 T G R L S C I D V D D C E A G K V C 774  
 CAA GAT GGC ATC TGC ACG AAC ACA CCA GGC TCT TTC CAG TGT CAG TGC CTC TCC 2376  
 Q D G I C T N T P G S F Q C Q C L S 792  
 GGC TAT CAT CTG TCA AGG GAT CGG AGC CGC TGT GAG GAC ATT GAT GAA TGT GAC 2430  
 G Y H L S R D R S R C E D I D E C D 810  
 TTC CCT GCG GCC TGC ATC GGG GGT GAC TGC ATC AAT ACC AAT GGT TCC TAC AGA 2484  
 F P A A C I G G D C I N T N G S Y R 828  
 TGT CTC TGT CCC CTG GGT CAT CGG TTG GTG GGC GGC AGG AAG TGC AAG AAA GAT 2538  
 C L C P L G H R L V G G R K C K K D 846  
 ATA GAT GAG TGC AGC CAG GAC CCA GGC CTG TGC CTG CCC CAT GCC TGC GAG AAC 2592  
 I D E C S Q D P G L C L P H A C E N 864  
 CTC CAG GGC TCC TAT GTC TGT GTC TGT GAT GAG GGT TTC ACA CTC ACC CAG GAC 2646  
 L Q G S Y V C V C D E G F T L T Q D 882  
 CAG CAT GGG TGT GAG GAG GTG GAG CAG CCC CAC CAC AAG AAG GAG TGC TAC CTT 2700  
 Q H G C E E V E Q P H H K K E C Y L 900  
 AAC TTC GAT GAC ACA GTG TTC TGT GAC AGC GTA TTG GCT ACC AAT GTC ACT CAG 2754  
 N F D D T V F C D S V L A T N V T Q 918  
 CAG GAA TGC TGT TGC TCT CTG GGA GCT GGC TGG GGA GAC CAC TGC GAA ATC TAT 2808  
 Q E C C C S L G A G W G D H C E I Y 936  
 CCC TGT CCA GTC TAC AGC TCA GCC GAA TTT CAC AGC CTG GTG CCT GAT GGG AAA 2862  
 P C P V Y S S A E F H S L V P D G K 954  
 AGG CTA CAC TCA GGA CAA CAA CAT TGT GAA CTA TGC ATT CCT GCC CAC CGT GAC 2916  
 R L H S G Q Q H C E L C I P A H R D 972  
 ATC GAC GAA TGC ATA TTG TTT GGG GCA GAG ATC TGC AAG GAG GGC AAG TGT GTG 2970  
 I D E C I L F G A E I C K E G K C V 990  
 AAC TCG CAG CCC GGC TAC GAG TGC TAC TGC AAG CAG GGC TTC TAC TAC GAT GGC 3024  
 N S Q P G Y E C Y C K Q G F Y Y D G 1008  
 AAC CTG CTG GAG TGC GTG GAC GTG GAC GAG TGC TTG GAT GAG TCT AAC TGC AGG 3078  
 N L L E C V D V D E C L D E S N C R 1026  
 AAC GGA GTG TGT GAG AAC ACG TGG CGG CTA CCG TGT GCC TGC ACT CCG CCG GCA 3132  
 N G V C E N T W R L P C A C T P P A 1044  
 GAG TAC AGT CCC GCA CAG GCC CAG TGT CTG AGC CCG GAG GAG ATG GAG CAC GCC 3186  
 E Y S P A Q A Q C L S P E E M E H A 1062

FIG. 25-3

SUBSTITUTE SHEET (RULE 26)

38/45

CCA GAG AGA CGT GAA GTG TGC TGG GGC CAG CGA GGA GAG GAC GGC ATG TGT ATG 3240  
 P E R R E V C W G Q R G E D G M C M 1080  
 GGG CCC CTG GCG GGA CCT GCC CTC ACT TTT GAT GAC TGC TGC TGC CGC CAG CCG 3294  
 G P L A G P A L T F D D C C C R Q P 1098  
 CGG CTG GGG TAC CAG TGC AGA CCG TGC CCG CCA CGT GGC ACC GGG TCC CAG TGC 3348  
 R L G Y Q C R P C P P R G T G S Q C 1116  
 CCG ACT TCA CAG AGT GAG AGC AAT TCT TTC TGG GAC ACA AGC CCC CTG CTA CTG 3402  
 P T S Q S E S N S F W D T S P L L L 1134  
 GGG AAG TCT CCG CGA GAC GAA GAC AGC TCA GAG GAG GAT TCA GAT GAG TGC CGT 3456  
 G K S P R D E D S S E E D S D E C R 1152  
 TGT GTG AGC GGA CCG TGT GTG CCA CGG CCA GGC GGG GCG GTA TGC GAG TGT CCT 3510  
 C V S G P C V P R P G G A V C E C P 1170  
 GGA GGC TTT CAG CTG GAC GCC TCC CGT GCC CGC TGC GTG GAC ATT GAT GAG TGC 3564  
 G G F Q L D A S R A R C V D I D E C 1188  
 CGA GAA CTG AAC CAG CGG GGA CTG CTG TGT AAG AGC GAG CGG TGC GTG AAC ACC 3618  
 R E L N Q R G L L C K S E R C V N T 1206  
 AGT GGA TCC TTC CGC TGT GTC TGC AAA GCT GGC TTC ACG CGC AGC CGC CCT CAC 3672  
S G S F R C V C K A G F T R S R P H 1224  
 GGG CCT GCG TGC CTC AGC GCC GCC GCT GAT GAT GCA GCC ATA GCC CAC ACC TCA 3726  
 G P A C L S A A A D D A A I A H T S 1242  
 GTG ATC GAT CAT CGA GGG TAT TTT CAC TGA  
 V I D H R G Y F H \*

FIG. 25-4

SUBSTITUTE SHEET (RULE 26)

39/45

Met Arg Gln Ala Ala Leu Gly Leu Leu Leu Ala Leu Leu Leu Ala Leu Gly Pro Gly Gly Arg 22  
 Gly Val Gly Arg Pro Gly Ser Gly Ala Gln Ala Gly Ala Gly Arg Trp Ala Gln Arg Phe Lys Val 44  
 Val Phe Ala Pro Val Ile Cys Lys Arg Thr Cys Leu Lys Gly Gln Cys Arg Asp Ser Cys Gln Gln 66  
 Gly Ser Asn Met Thr Leu Ile Gly Leu Ile Gly Glu Asn Gly His Ser Thr Asp Thr Leu Thr Gly Ser Ala Phe 88  
 Arg Val Val Val Cys Pro Leu Pro Cys Met Asn Gly Gly Gln Cys Ser Ser Arg Asn Gln Cys Leu 110  
 Cys Pro Pro Asp Phe Thr Gly Arg Phe Cys Gln Val Pro Ala Ala Gly Thr Gly Ala Gly Thr Gly 132  
 Ser Ser Gly Pro Gly Trp Pro Asp Arg Ala Met Ser Thr Gly Pro Leu Pro Pro Leu Ala Pro Glu 154  
 Gly Glu Ser Val Ala Ser Lys His Ala Ile Tyr Ala Val Gln Val Ile Ala Asp Pro Pro Gly Pro 176  
 Gly Glu Gly Pro Pro Ala Gln His Ala Ala Phe Leu Val Pro Leu Gly Pro Gly Gln Ile Ser Ala 198  
 Glu Val Gln Ala Pro Pro Pro Val Val Asn Val Arg Val His His Pro Pro Glu Ala Ser Val Gln 220  
 Val His Arg Ile Glu Gly Pro Asn Ala Glu Gly Pro Ala Ser Ser Gln His Leu Pro His Pro 242  
 Lys Pro Pro His Pro Arg Pro Pro Thr Gln Lys Pro Leu Gly Arg Cys Phe Gln Asp Thr Leu Pro 264  
 Lys Gln Pro Cys Gly Ser Asn Pro Leu Pro Gly Leu Thr Lys Gln Glu Asp Cys Cys Gly Ser Ile 286  
 Gly Thr Ala Trp Gly Gln Ser Lys Cys His Lys Cys Pro Gln Leu Gln Tyr Thr Gly Val Gln Lys 308  
 Pro Val Pro Val Arg Gly Glu Val Gly Ala Asp Cys Pro Gln Gly Tyr Lys Arg Leu Asn Ser Thr 330  
 His Cys Gln Asp Ile Asn Glu Cys Ala Met Pro Gly His Ser Leu Cys His Gly Asp Cys Leu Asn Asn 352  
 Pro Gly Ser Tyr Arg Cys Val Cys Pro Pro Gly His Ser Leu Gly Pro Leu Ala Ala Gln Cys Ile 374  
 Ala Asp Lys Pro Glu Glu Lys Ser Leu Cys Phe Arg Leu Val Ser Thr Glu His Gln Cys Gln His 396  
 Pro Leu Thr Thr Arg Leu Thr Arg Gln Leu Cys Cys Ser Val Gly Lys Ala Trp Gly Ala Arg 418  
 Cys Gln Arg Cys Pro Ala Asp Gly Thr Ala Ala Phe Lys Glu Ile Cys Pro Gly Trp Glu Arg Val 440  
 Pro Tyr Pro His Leu Pro Pro Asp Ala His His Pro Gly Gly Lys Arg Leu Pro Leu Pro Ala 462  
 Pro Asp Gly Pro Pro Lys Pro Pro Gln Leu Pro Glu Ser Pro Ser Arg Ala Pro Pro Leu Glu Asp 484

FIG. 26-1

40/45

Thr Glu Glu Glu Arg Gly Val Thr Met Asp Pro Pro Val Ser Glu Glu Arg Ser Val Gln Gln Ser 506  
 His Pro Thr Thr Thr Thr Ser Pro Pro Arg Pro Tyr Pro Glu Leu Ile Ser Arg Pro Ser Pro Pro 528  
 Thr Phe His Arg Phe Leu Pro Asp Leu Pro Pro Pro Ser Arg Ser Ala Val Glu Ile Ala Pro Thr Gln 550  
 Val Thr Glu Thr Asp Glu Cys Arg Leu Asn Gln Asn Ile Cys Gly His Gly Gln Cys Val Pro Gly 572  
 Pro Ser Asp Tyr Ser Cys His Cys Asn Ala Gly Tyr Arg Ser His Pro Gln His Arg Tyr Cys Val 594  
 Asp Val Asn Glu Cys Glu Ala Glu Pro Cys Gly Pro Gly Lys Gly Ile Cys Met Asn Thr Gly Gly 616  
 Ser Tyr Asn Cys His Cys Asn Arg Gly Tyr Arg Leu His Val Gly Ala Gly Arg Ser Cys Val 638  
 Asp Leu Asn Glu Cys Ala Lys Pro His Leu Cys Gly Asp Gly Gly Phe Cys Ile Asn Phe Pro Gly 660  
 His Tyr Lys Cys Asn Cys Tyr Pro Gly Tyr Arg Leu Lys Ala Ser Arg Pro Pro Ile Cys Glu Asp 682  
 Ile Asp Glu Cys Arg Asp Pro Ser Thr Cys Pro Asp Gly Lys Cys Glu Asn Lys Pro Gly Ser Phe 704  
 Lys Cys Ile Ala Cys Gln Pro Gly Tyr Arg Ser Gln Gly Gly Ala Cys Arg Asp Val Asn Glu 726  
 Cys Ser Glu Gly Thr Pro Cys Ser Pro Gly Trp Cys Glu Lys Leu Pro Gly Ser Tyr Arg Cys Thr 748  
 Cys Ala Gln Gly Ile Arg Thr Arg Thr Gly Arg Leu Ser Cys Ile Asp Val Asp Cys Glu Ala 770  
 Gly Lys Val Cys Gln Asp Gly Ile Cys Thr Asn Thr Pro Gly Ser Phe Gln Cys Cys Leu Ser 792  
 Gly Tyr His Leu Ser Arg Asp Arg Ser Arg Cys Glu Asp Ile Asp Glu Cys Asp Phe Pro Ala Ala 814  
 Cys Ile Gly Gly Asp Cys Ile Asn Thr Asn Gly Ser Tyr Arg Cys Leu Cys Pro Leu Gly His Arg 836  
 Leu Val Gly Gly Arg Lys Cys Lys Asp Ile Asp Glu Cys Ser Gln Asp Pro Gly Leu Cys Leu 858  
 Pro His Ala Cys Glu Asn Leu Gln Gly Ser Tyr Val Cys Val Cys Asp Glu Gly Phe Thr Leu Thr 880  
 Gln Asp Gln His Gly Cys Glu Glu Val Glu Gln Pro His His Lys Lys Glu Cys Tyr Leu Asn Phe 902  
 Asp Asp Thr Val Phe Cys Asp Ser Val Leu Ala Thr Asn Val Thr Gln Gln Glu Cys Cys Ser 924  
 Leu Gly Ala Gly Trp Gly Asp His Cys Glu Ile Tyr Pro Cys Pro Val Tyr Ser Ser Ala Glu Phe 946  
 His Ser Leu Val Pro Asp Gly Lys Arg Leu His Ser Gly Gln Gln His Cys Glu Leu Cys Ile Pro 968

FIG. 26-2

41/45

Ala His Arg Asp Ile Asp Glu Cys Ile Leu Phe Gly Ala Glu Ile Cys Lys Glu Gly Lys Cys Val	990
Asn Ser Gln Pro Gly Tyr Glu Cys Tyr Cys Lys Gln Gly Phe Tyr Tyr Asp Gly Asn Leu Glu	1012
Cys Val Asp Val Asp Glu Cys Thr Pro Pro Ala Glu Ser Asn Cys Arg Asn Gly Val Cys Glu Asn Thr Trp	1034
Arg Leu Pro Cys Ala Cys Thr Pro Gln Arg Asp Val Lys Cys Ala Gly Ala Ser Glu Gln Cys Leu Ile Pro	1056
Glu Arg Trp Ser Thr Pro Gln Arg Asp Val Lys Cys Ala Gly Ala Ser Glu Glu Arg Thr Ala Cys	1078
Val Trp Gly Pro Trp Ala Gly Pro Ala Leu Thr Phe Asp Asp Cys Cys Arg Gln Pro Arg Leu	1100
Gly Thr Gln Cys Arg Pro Cys Pro Pro Arg Gly Thr Gly Ser Gln Cys Pro Thr Ser Gln Ser Glu	1122
Ser Asn Ser Phe Trp Asp Thr Ser Pro Leu Leu Ser Gly Lys Ser Pro Arg Asp Glu Asp Ser Ser	1144
Glu Glu Asp Ser Asp Glu Cys Arg Phe Gln Leu Asp Ala Ser Arg Ala Arg Cys Val Arg Pro Gly Ala Val	1166
Cys Glu Cys Pro Gly Gly Arg Gly Phe Gln Leu Cys Lys Ser Arg Ala Arg Cys Val Asp Ile Asp Glu Cys	1188
Arg Glu Leu Asn Gln Arg Gly Leu Leu Cys Lys Ser Glu Arg Cys Val Asn Thr Ser Gly Ser Phe	1210
Arg Cys Val Cys Lys Ala Gly Phe Thr Arg Ser Arg Pro His Gly Pro Ala Cys Leu Ser Ala Ala	1232
Ala Asp Asp Ala Ala Ile Ala His Thr Ser Val Ile Asp His Arg Gly Tyr Phe His	1251

FIG. 26-3

42/45

ATGGAGAGCA CCTCCCCGCG AGGTCTCCGG TGCCACACAGC TCTGCAGCCA CTCTGGGGCC ATGAGAGCGC CGACCACCGC 80  
 TCGTGTCTCC GGATGCATCC AACGGGTGCG TTGGAGGGCG TTCTTGCCAC TTGTCTTGCG TGTCTTGATG GGGACAAGTC 160  
 ATGCCCAACG GGATTCCATA GGGAGATACG AACAGCTAG CAGGATGCG AATCGGTTGT GGCACCCCGT GGGCAGCCAC 240  
 CCGCAGCGG CTGCAGCCAA GGTGTACAGT CTGTTCGAG AGCTGACGC GCCGGTCCCC GGTGTGTCGC CCTCTGAGTG 320  
 GAACCAAGCG GCCCAGGGA ACCCGGATG GCTCGCAGAG GCCGAGGCCA GGAGGCCACC TCGAACCCAG CAGCTGCGTC 400  
 GAGTCCAGCC ACCTGTCCAG ACTCGGAGAA GCCATCCCCG GTGCAACGGC GAGGGCGGT CACTGGGAGA AATGTCTGCG GGGGACAGTG 480  
 GCGCGCCTGG AAACCCCTCA GCGACCCGCG GCTGCACGGC CAACCACTGT ATCAAACTTG TGTGTACGCC TCCCTGTGAG AACCGAGGCT 560  
 CTGCCCCAGG TGGACAACAT CAAAACAGCAC TGCACTCTGCC GTTCTGGCTT CCGTGGGGCG AGGTCAATCCC TGAGGAGGAA 640  
 CCTGCAAGCAG GCCCCAGGTC TGCATCTGCC GCCTGTGCCC AGACGCTCAG TGGAGAGAGC ACCGGTCCT CACAGAAGCA GTGAGGCCAG 720  
 TTTGACCCCTC AGAATGCCAG GCCTGTGCCC TACAGCCGCT GGTACCACCA CCATCACAC CTTCCATCTCG GCGCCTCAGC CAGCCCTGGC 800  
 AGGAAGTCTA GTGACCAGAA TACAGCCGCT GGTACCACCA CCATCACAC CTTCCATCTCG GCGCCTCAGC CAGCCCTGGC 880  
 CCTGTCAGCA GCACTCAGGG CCGTCCAGGA CAGTTCTGTCG GTATCCGGCC ACTGGTGCCA ATGGCCAGCT GATGTCCAAC 960  
 GCTTTGCTTT CAGGACTCGA GCTGAGAGAC AGCAGCCAC AGCAGCACA TGTGAACCAT CTCTCACCCC CCTGGGGCT 1040  
 GAACCTCACC GAGAAAATCA AGAAATCAA AGTCGTCTTC ACCCCACCA TGTGCAAGCA GACCTGTGCC CGGGGACGCT 1120  
 GTGCCAACAG CTGTGAGAAG GGTGACACCA CCACCTTGTA CAGTCAGGT GGCATGGGC ATGACCCCAA GTCTGGCTTC 1200  
 CGTATCTATT TCTGCCAAT CCCCTGCCTG AATGTGGCC GCTGCATCGG CCGGGACGAG TGTGTGTGTC CAGCCAATC 1280  
 CACAGGAAAG TTCTGCCATC TGCCCTGTCCC GCAGCCAGAC AGGAAACCTG CAGGGCGAGG TTCCCGGCAC AGAACCCCTGC 1360  
 TGGAAAGTCC CCTGAAGCAA TCCACCTTCA CGCTGCCCTCT CTCTAACCCAG CTCGCCCTCTG TGAACCCCTC GCTGGTGAAG 1440  
 GTGCAAAATC ATCACCCGCC TGAGGCCCTCT GTGCAGATTC ACCAGGTGCC CCGGGTCCGG GGTGAGCTGG ACCCCGTGCT 1520  
 GGAGGACAAC AGTGTGGAGA CCAGAGCCTC TCATCGCCCC CACGGCAACC TAGGCCACAG CCCCTGGGCC AGCAACAGCA 1600  
 TACCCGCTCG GGCCGGAGAG GCCCCTCGGC CACCACCAGT GCTGTCTAGG CATTATGGAC TTCTGGGCCA GTGTTACCTG 1680  
 AGCACGGTGA ATGGACAGTG TGCTAACCCC CTAGGTAGTC TGACTTCTCA GGAGGACTGC TGTGGCAGTG TGGGACCTT 1760  
 CTGGGGGGTG ACCTCCTGTG CTCCCTGCCC ACCCAGACAA GAGGGTCCAG CCTTCCAGT GATTGAAAAAT GGCCAGCTGG 1840  
 AGTGTCCCCA AGGATAACAAG AGACTGAACC TCAGCCACTG CCAAGATATC AATGAGTGCC TGACCCCTGGG CCTCTGCAAG 1920

FIG. 27-1

43/45

GACTCGGAGT GCGTGAAACAC CAGGGGCAGC TACCTGTGCA CCTGCAGGCC TGGCCTCATG CTGGATCCGT CAAGGAGCCG 2000  
CTGCGTATCG GACAAGGCTG TCTCCATGCA GCAGGGACTA TGCTACCGGT CACTGGGGTC TGGTACCTGC ACCCTGCTTT 2080  
TGGTTTCATCG GATCACCAAG CAGATATGCT GCTGCAGCCG TGTGGGCAAA GCCTGGGGTA GCACATGTGA ACAGTGTCCC 2160  
CTGCCTGGCA CAGAAGCCTT CAGGGAGATC TGCCCTGTG GGCATGGCTA CACCTACTCG AGCTCAGACA TCCGCCCTGTC 2240  
TATGAGGAAA GCCGAAGAAG AGGAACTGGC TAGCCCTTTA AGGAGCAGA CAGAGCAGAG CACTGCACCC CCACCTGGGC 2320  
AAGCAGAGAG GCAACCACTC CGGGCAGCCA CCGCCACCTG GATTGAGGCT GAGACCTCC CTGACAAAAGG TGACTCTCGG 2400  
GCTGTTTCAGA TCACAAACCAG TGCTCCCCAC CTACCTGCCC GGTACCAGG GGATGCCACT GGAAGACCAG CACCATCCTT 2480  
GCCTGGACAG GGCATTCCAG AGAGTCCAGC AGAAGAGCAA GTGATTCCCT CCAGTGATGT CTTGGTGACA CACAGCCCCC 2560  
CAGACTTTGA TCCATGTTTT GCTGGAGCCT CCAACATCTG TGGCCCTGGG ACCTGTGTGA GCCTCCCAA TGGATACAGA 2640  
TGTGTCGCA GCCCTGGCTA CCAGCTACAC CCCAGCCAAG ACTACTGTAC TGATGACAAC GAGTGTATGA GGAACCCCTG 2720  
TGAAGGAAGA GGGCGCTGTG TCAACAGTGT GGGCTCCTAC TCCTGCCCTT GCTATCCTGG CTACACACTA GTCACCTCG 2800  
GAGACACACA GGAGTGCCAA GATATCGATG AGTGTGAGCA GCCCGGGTG TGCACTGGTG GCGGATGCAG CAACACGGAG 2880  
GGCTCGTACC ACTGCGAGTG TGATCGGGG TACATCATGG TCAGGAAAGG ACACTGTCAA GATATCAACG AATGCCGTCA 2960  
CCCTGGTACC TGCCCTGATG GGAGATGCGT CAACTCCCCT GGCTCCTACA CTTGTCTGGC CTGTGAGGAG GGCTATGTAG 3040  
GCCAGAGTGG GAGCTGTGTA GATGTCAATG AGTGTCTGAC CCCTGGGATA TGTAACCATG GAAGGTGCAT CAACATGGAA 3120  
GGCTCCTTTA GATGCTCCTG TGAGCCGGGC TATGAGGTCA CCCCAGACAA GAAAGGCTGC CGAGATGTGG ACGAGTGTGC 3200  
CAGCCGAGCC TCGTGCCCCA CGGGCCTCTG CCTCAACACG GAGGGCTCCT TCACCTGCTC AGCCTGTGAG AGCGGTACT 3280  
GGGTGAACGA AGATGGCACT GCTGTGAAG ACTTGGATGA ATGTGCCCTC CCTGGAGTCT GCCCCACAGG CGTCTGCACC 3360  
AATACTGTAG GCTCCTTCTC CTGCAAGGAC TGTGACCAGG GCTACCGGCC CAACCCCTG GGCAACAGAT GCGAAGATGT 3440  
GGATGAGTGT GAAGGTCCCC AAAGCAGCTG CCGGGGAGGC GAATGCAAGA ACACAGAAGG TTCTACCAA TGCCTCTGTC 3520  
ACCAGGGCTT CCAGCTGGTC AATGGCACCA TGTGTGAGGA CGTGAATGAG TGTGTTGGGG AAGAGCATTG TGCTCCTCAC 3600  
GGCGAGTGCC TCAACAGCCT GGGCTCCTTC TTCTGCCCTCT GTGCACCCGG CTTTGTAGT GCTGAGGGGG GCACCAGATG 3680  
CCAGGATGTT GATGAATGTG CAGCCACAGA CCCGTGTCCG GGAGGACACT GTGTCAACAC AGAGGGCTCC TTCAGCTGTC 3760  
TGTGTGAGAC TGCTTCCTTC CAGCCCTCCC CAGACAGCGG AGAATGTTTG GATATTGATG AGTGTGAGGA CCGTGAAGAC 3840

FIG. 27-2

44/45

CCGGTGTGCG GAGCCTGGAG GTGTGAGAAC AGTCCTGGTT CCTACCGCTG CATCCTGGAC TGCCAGCCTG GATTCTATGT 3920  
GGCGCCAAAT GGAGACTGCA TTGACATAGA TGAATGTGCC AATGACACTG TGTGTGGGAA CCATGGCTTC TGTGACAAACA 4000  
CGGACGGCTC CTTCCGCTGC CTGTGTGACC AGGGCTTCCA GACCTCACCA TCAGGCTGGG AGTGTGTGTA TGTGAACGAG 4080  
TGTGAGCTCA TGATGGCAGT GTGTGGGGAT GCGCTCTGTG AGAACGTGGA AGGCTCCTTC CTGTGCTTTT GCGCCAGTGA 4160  
CCTTGAGGAG TACGACGCAG AAGAAGGACA CTGCCGCTCT CGGGTGGCTG GAGCTCAGAG AATCCCAGAG GTCCGGACAG 4240  
AGGACCAGGC TCCAAGCCTT ATCCGCAATG AATGCTACTC TGAACACAAT GGTGCTCCTC CCTGCTCTCA AATCCTGGGC 4320  
CAGAACTCCA CACAGGCCGA GTGCTGCTGC ACTCAGGGTG CCAGATGGGG AAAGGCTGTG GCGCCCTGCC CATCTGAGGA 4400  
CTCAGTTGAA TTCAGTCAGC TCTGCCCCAG TGGTCAAGGT TACATCCCAG TGAAGGAGC CTGGACATTT GGACAAACCA 4480  
TGTATACAGA TGCCGATGAA TGTGTACTGT TTGGGCTGTC TCTCTGCCAG AATGGCCGAT GCTCAAACAT AGTGCCCTGGC 4560  
TACATTTGCC TGTGCAACCC TGGCTACCAC TATGATGCCT CCAGCAGGAA GTGCCAGGAT CACAACGAAT GCCAGGACTT 4640  
GGCCTGTGAG AACGGTGAGT GTGTGAACCA AGAAGGCTCC TTCCATTGCC TCTGCAATCC CCCCCTCAC CTAGACCTCA 4720  
GTGGGCAGCG CTGTGTGAAC ACGACCAGCA GCACGGAGGA CTTCCCTGAC CATGACATCC ACATGGACAT CTGCTGGAAA 4800  
AAAGTCACCA ATGATGTGTG CAGCCAGCCC TTGCGTGGGC ACCATACCAC CTATACAGAA TGCTGCTGCC AAGATGGGGA 4880  
GGCCTGGAGC CAGCAATGCG CTCTGTGCCC GCCCAGGAGC TCTGAGGTCT ACGCTCAGCT GTGCAACGTG GCTCGGATTG 4960  
AGGCAGAGCG CGGAGCAGGG ATCCACTTCC GGCCAGGCTA TGAGTATGGC CCTGGCCTGG ACGATCTGCC TGAATAACCTC 5040  
TACGGCCCGAG ATGGGGCTCC CTTCTATAAC TACCTAGGCC CCGAGGACAC TGCCCTGAG CCTCCCTTCT CCAACCCAGC 5120  
CAGCCAGCCG GGAGACAAACA CACCTGTCTT TGAGCCTCCT CTGCAGCCCT CTGAACTTCA GCCTCACTAT CTAGCCAGCC 5200  
ACTCAGAACCC CCTGCTCTCC TTCGAAGGCC TTCAGGCTGA GGAATGTGGC ATCCTGAATG GCTGTGAGAA TGGCCGCTGC 5280  
GTGCGTGTGC GGGAGGGCTA CACTTGGGAC TGCTTTGAGG GCTTCCAGCT GGATGCGCCC ACATTGGCCT GTGTGGATGT 5360  
GAACGAGTGT GAAGACTTGA ACGGGCCTGC ACGACTCTGT GCACACGGTC ACTGTGAGAA CACAGAGGGT TCCTATCGCT 5440  
GCCACTGTTC GCCAGTTAC GTGGCAGAGC CAGGCCCCCC AACTGTGCG GCCAAGGAGT AG 5502



45/45

90      MESTSPRGLRCPQLCSHSGAMRAPTTARCSGCIQVRWRGFLPLVLAVLMTSHAQRDSIGRYEPASRDANRLWHPVGSHPAAAAKVYS  
 180      LFREPDAPVPLSPSEMNQPAQGNPGWLAEAEARRPRTQQLRRVQPPVQTRRSHPRGQQQIAARAAPSVARLETTPQRAAARRGRLTGR  
 270      NVCGGQCCPWTTSNTHCIKPVQPPCQNRGSCSRPQVCICRSGFRGARCCEEVIPEEEFDPQNAARPVRRSVERAPGPHRSSEARGSL  
 360      VTRIQLVPPPPSPRRRLSQPWPLQQHSGPSRTVRRYPATGANGQLMSNALPSGLELRDSSPQAAHVNHLSPPWGLNLTEKIKKIKVVF  
 450      TPTICKQTCARGRCANSCKEKGDTTTLYSQGGHGHDPKSGFRIYFCQIPCLNGGRCIGRDECWCPANSTGKFCFCHLPVPQPDREPAGRSRH  
 540      RTLLEGPLKQSTFTLPLSNQLASVNPISLVKVQIHHPPEASVQIHQVARVRGELDPVLEDNSVETRASHRPHGNLGHSPWASNSIPARAGE  
 630      APRPPFVLSRHYGLLGQCYLSTVNGQCANPLGSLTSQEDCCGSVGTFWGVTSCAPCPPRQEGPAPFVIENGQLECPQGYKRLNLSHCQDI  
 720      NECLTLGLCKDSECVNTRGSYLCTCRPGLMLDPSRRCVSDKAVSMQQGLCYRSLSGTCTTLPLVHRITKQICCCSRVKGAWGSTCEQCP  
 810      LPGTEAFREICPAGHGYTSSDIRLSMRKAEELASPLREQTEQSTAPPPQAEQRLRAATATWIEAETLPDKGDSRAVQITTSAPH  
 900      LPARVPGDATGRPAPSLPGQGIPESPAEEQVIPSDDVLVTHSPDFPCFAGASNICGPGTCVSLPNGYRCVCSPGYQLHPSQDYCTDDN  
 990      ECMRNPCEGRGRCVNSVGSYSCLCYPGYTLVTLGDTQECQDIDECEQPGVCSGGRCNSNTEGSHCECDRGYIMVRKGHCQDINECRHPGT  
 1080      CPDGRCVNSPGSYTCLACEGYVQSGSCVDVNECLTPGICTHGRGINMEGSFRCSCEPGYEVTPDKKGC RDVDECA SRASCPTGLCLNT  
 1170      EGSFTCSACQSGYWNEDGTACEDLDECAFPVCPTGVCINTVGSFCKDCDQGYRPNPLGNRCEDVDECEGPQSSCRGGECKNTEGSYQ  
 1260      CLCHQGFQLVNGTMCEDVNECVGEEHCAPHGECLNSLGSFFCLCAPGFASAEGRTRCQDVDECAATDPCPGGHCVNTEGSFSCLCETASF  
 1350      QPSPDSGECILDIDECEDREDPVCGAWRCENSPGSYRCILDCQPGFYVAPNGDCIDIDECANDTVCGNHGFCNDTSGFRCLCDQGFETSP  
 1440      SGWECVDVNECELMMAVCGDALCENVEGSFLCLCASDLEEDAEEGHCRPRVAGAQRIPVVRTEDQAPSLIRMECYSEHNGGPPCSQILG  
 1530      QNSTQAECCCTQGARWGKACAPCPSEDSVEFSQLCPSGQGYIPVEGAWTFGQTMYTDADECVLFGPALCQNGRCSNIVPGYICLCNPGYH  
 1620      YDASSRKCQDHNECCQDLACENGECVNQEGSFHCLCNPPLTLDLSGQRCVNTTSSSTEDFPDHD IHMDICWKKVTVNDVCSQPLRGHHTTYTE  
 1710      CCCQDGEAWSQQCALCPPRSSEVYAQLCNVARI EAERGAGIHFRPGYEGPGLDDL PENLYGPDGAPFYNYLGPEDTAP EPPFSNPASQP  
 1800      GDNTPVLEPPLQPSSELQPHYLASHSEPPASFEGLOAECCGILNGCENGRCVRREGYTCDFEGFQLDAPT LACVDVNECEDLNGPARLC  
 1833      AHGHCNTEGSRCHCSPGYVAEPGPHCAAKE

FIG. 28



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/12, 15/16, A61K 48/00, 38/39, C07K 14/47, A61L 27/00</b>		<b>A3</b>	(11) International Publication Number: <b>WO 95/22611</b> (43) International Publication Date: 24 August 1995 (24.08.95)
(21) International Application Number: PCT/US95/02251 (22) International Filing Date: 21 February 1995 (21.02.95)  (30) Priority Data: 08/199,780 18 February 1994 (18.02.94) US 08/316,650 30 September 1994 (30.09.94) US  (71) Applicant: THE REGENTS OF THE UNIVERSITY OF MICHIGAN [US/US]; Wolverine Tower, Room 2071, 3003 South State Street, Ann Arbor, MI 48109-1280 (US). (74) Agent: PARKER, David, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).			(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  (88) Date of publication of the international search report: 8 February 1996 (08.02.96)
(54) Title: METHODS AND COMPOSITIONS FOR STIMULATING BONE CELLS			
(57) Abstract  Disclosed are methods, compositions, kits and devices for use in transferring nucleic acids into bone cells <i>in situ</i> and/or for stimulating bone progenitor cells. Type II collagen and, particularly, osteotropic genes, are shown to stimulate bone progenitor cells and to promote bone growth, repair and regeneration <i>in vivo</i> . Gene transfer protocols are disclosed for use in transferring various nucleic acid materials into bone, as may be used in treating various bone-related diseases and defects including fractures, osteoporosis, osteogenesis, imperfecta and in connection with bone implants.			

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## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N15/16 A61K48/00 C07K14/47 A61K38/39  
A61L27/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C12N A61L C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 05199 (GENETICS INSTITUTE) 2 April 1992  see page 12 - page 15 ---	17,26, 65, 77-128
A	DE,A,42 19 626 (WEHLING) 23 December 1993 see the whole document ---	1
A	TRENDS IN GENETICS, vol.8, no.3, pages 97 - 102 V. ROSEN ET AL. 'The BMP proteins in bone formation and repair' see the whole document ---	1
A	WO,A,93 05751 (CREATIVE BIOMOLECULES) 1 April 1993 see the whole document ---	1
-/--		



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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6

Date of the actual completion of the international search

27 July 1995

Date of mailing of the international search report

29.12.95

Name and mailing address of the ISA

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Authorized officer

SKELLY J.M.

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 01139 (BAYLOR COLLEGE OF MEDICINE) 20 January 1994	1-16, 18-25, 59-64, 66-76
Y	see the whole document  -----	17,26, 65, 77-128

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 2,3,27,28,149  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although these claims are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search been carried out and based on the alleged effects of the compound/ composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. claims 1-128
2. claims 129-131
3. claims 132-155

See continuation sheet PCT/ISA/210

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-128

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/SAJ/210

1. Compositions devices and methods for therapy of bone diseases comprising nucleic acid
2. Latent TGF-beta binding protein-3 (LTBP-3) and DNA encoding it
3. Compositions devices and methods for therapy of bone diseases comprising Type II collagen.

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		JP-T- 7508988	05-10-95



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